

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 391

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ON SEROTONIN AND CATECHOLAMINE
NEURONS IN THE RAT CNS

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UNIVERSITY OF HAMBURG, G.F.R. AND DEPARTMENT OF HISTOLOGY
UNIVERSITY OF LUND SWEDEN

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Abbreviations used 5,7-DHT 5,7-dihydroxytryptamine 5,8-DHT 5,8-dihydroxytryptamine 5-HT 5-hydroxytryptamine (serotonin); CA, catecholamines NA, noradrenaline; DA dopamine; IA, indolamines.

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SUMMARY

Doses of 10–200 μg (free base) of 5,7-dihydroxytryptamine (5,7-DHT) injected into the lateral ventricle induced a dose-dependent significant long lasting reduction of serotonin and noradrenaline in rat brain and spinal cord. The time-course of depletion and recovery of brain serotonin was analysed in individual brain regions between 3 hrs and 45 days after a single high dose of 5,7-DHT (200 μg). In ventricle near regions (septum and hypothalamus) as well as in the cell body rich parts of the rat CNS (mesencephalon and pons + medulla oblongata) a profound reduction in 5-HT (up to 80 %) was registered by 3–24 hrs. After a transient recovery phase with somewhat higher 5-HT concentrations at 4 days (35–55 % of control) the 5-HT levels declined to near minimum values (15–45 % of control) at 10 days. Thereafter a slight non-significant recovery of 5-HT occurred between 10 and 45 days. Spinal cord, the forebrain-rost sample and striatum differed in their response to 5,7-DHT from the other brain regions, and revealed a more gradual and retarded loss of 5-HT which resulted in minimum values (10–25 % of control) at 10 days. The levels of serotonin then remained depressed up to 45 days.

10 days after 10–200 μg 5,7-DHT whole brain dopamine concentrations did not differ from control. In contrast, whole brain and spinal cord noradrenaline levels showed a dose dependent reduction 10 days after 10–200 μg 5,7-DHT. Following a single high dose (200 μg) of 5,7-DHT there was a rapid reduction in spinal cord and whole brain noradrenaline to approximately 25 % of control at 3 hrs. During the subsequent four days there was a partial recovery in the brain noradrenaline concentration to 35–55 % of control at 4–45 days. In the spinal cord there was a transient recovery at 1–4 days, and maximum reduction (about 15 % of control) was reached by 10 and 45 days.

The long lasting depletion of brain and spinal cord 5-HT and noradrenaline concentrations was — at 9–11 days after 200 μg of 5,7-DHT — accompanied by a strong reduction in the ability of slices from brain and spinal cord to accumulate ^3H -5-HT (85–90 % reduction) and ^3H -noradrenaline (40–60 % reduction). The fluorescence histochemical observations revealed an almost complete long term disappearance of indolamine-containing terminals, and to a lesser extent also noradrenaline-containing terminals, in several regions of the brain and spinal cord. In addition, there were fluorescence microscopical signs of extensive axonal damage in the major indolamine and noradrenaline axon bundles.

The results provide evidence that 5,7-DHT has a neurotoxic mode of action on serotonin neurons, and to a lesser extent also on noradrenaline neurons in the CNS.

It seems now well documented that 5,6-dihydroxytryptamine (5,6-DHT) injected into the cerebrospinal fluid, causes degeneration of — primarily indolamine-containing axons and axon terminals. This neurotoxic mode of action of 5,6-DHT has been observed as a long-lasting depletion of brain and spinal cord serotonin levels (Baumgarten, Björklund, Lachenmayer, Nobin and Stenevi 1971; Baumgarten, Evetts, Holman, Iversen, Vogt and Wilson 1972) accompanied by ultrastructural signs of degeneration of non-myelinated indolamine-containing axons and axon terminals (Baumgarten, Björklund, Holstein and Nobin 1972) by a reduction of 5-HT uptake sites (Björklund, Nobin and Stenevi 1973 a, b; Daly, Fuxe and Johanson 1973) and by a disappearance of fluorescence histochemically detectable indolamine-containing axons and axon terminals (Baumgarten, Lachenmayer and Schlossberger 1972; Björklund, Nobin and Stenevi 1973 a, b; Nobin, Baumgarten, Björklund, Lachenmayer and Stenevi 1973). With doses of 5–50 μ g the degenerative effect of intraventricularly administered 5,6-DHT — with respect to the monoamine neuron systems at least — remarkably selective for the indolamine neurons. 5,6-DHT has been found to cause transient reduction in brain NA and DA levels (Baumgarten *et al.* 1971) but this depletory action of 5,6-DHT on CA neurons is only to a minor extent accompanied by axonal degeneration (Björklund *et al.* 1973 a, Nobin *et al.* 1973).

When administered intraventricularly however 5,6-DHT has definite limitations as a tool for total and selective destruction of the indolamine-containing axons in the brain. Above all, a good selectivity of its neurotoxic effects is only obtained with doses up to 50–75 μ g. With these doses an almost total destruction of the indolamine fibres is obtained only in the spinal cord, whereas in the brain the reduction in 5-HT and ³H-5-HT uptake varies between 35 % and 65 % in different regions (Baumgarten *et al.* 1971; Björklund *et al.* 1973 a, b). Doses higher than 75 μ g proved to be severely toxic to the animals, causing muscle paralysis, convulsions and tremor and most animals did not survive. Already after 75 μ g, and more pronounced after 100 μ g of 5,6-DHT the fluorescence and electron microscopic investigations have given evidence of a non-selective damage to myelinated fibre systems and a shrinkage of the striatum on the side of the injections, and there is a deposition of a brownish (probably melanin) pigment on the walls of the ventricles and the aqueduct (Baumgarten *et al.* 1972 a, b; Nobin *et al.* 1973). Björklund, Nobin and Stenevi (1973 c) have demonstrated that this limitation in the use of 5,6-DHT — when administered intraventricularly — can be overcome by injecting small amounts of the drug directly into the brain parenchyma. Furthermore, this mode of administration allows more complete denervations of many brain regions, and thus after injection of 4 μ g of 5,6-DHT directly into the ventromedial midbrain tegmentum (i.e. close to the major ascending

indolamine fibre bundles) forebrain 5-HT levels and ^3H 5-HT uptake were reduced by as much as 75 % and 60–70 % respectively (Bjorklund *et al.* 1973 c)

The discovery of the neurotoxic properties of 5,6-DHT prompted us to test other di- and monohydroxylated tryptamines as tools for chemical degeneration of central indolamine neurons. The drawbacks of 5,6-DHT seem at least partly to be related to its high tendency to become oxidized at physiological pH. The more stable isomere, 5,7-dihydroxytryptamine (5,7 DHT) should therefore be of particular interest, as the greater chemical stability of this compound should give it better diffusion properties and, perhaps, less general toxicity. Preliminary fluorescence microscopic (Baumgarten and Lachenmayer 1972) and biochemical studies (Baumgarten, Victor and Lovenberg 1973) have, in fact, provided evidence for a high neurotoxic potency of 5,7 DHT. In the present investigation the possible neurotoxic effect of 5,7 DHT on monoamine-containing neurons have been evaluated in greater detail in the rat CNS.

MATERIAL AND METHODS

Adult female albino rats (180–220 g) were given single injections of 10–200 μg free base of 5,7-dihydroxytryptamine (creatinine sulfate H_2O complex) into the lateral ventricle, under ether anaesthesia. The drug was dissolved in sterile saline containing 1.0 mg/ml ascorbic acid. The injection volume was 20 μl . In order to protect the animals from convulsions, they were treated with 40 mg/kg, *s.p.* of Nembutal® (Abbot) immediately after the 5,7 DHT injection. Nembutal was required only for animals receiving more than 50 μg 5,7 DHT. The animals were killed by decapitation at times varying between 45 min and 45 days after the administration of 5,7 DHT.

Monoamine determinations were carried out on either whole brains (olfactory bulbs and pineal gland removed) and spinal cord, or on pooled brain regions, dissected according to Baumgarten *et al.* (1971). 5-Hydroxytryptamine (5-HT) was determined fluorometrically according to Bertler (1961) and noradrenaline (NA) and dopamine (DA) according to Bertler, Carlsson, Rosengren and Waldeck (1958) as modified by Haggendal (1963).

For the regional determinations of 5-HT regions from 4–5 rats were pooled for each sample. The number of samples assayed per time point were 8 samples from control animals, 4 samples from animals with a survival time of 3 and 24 hrs, and 4, 10 and 30 days, and 2 samples from 45-day animals. In the dose-response experiments 5-HT was determined on single whole brains and spinal cords, 4–5 animals being used for each point. NA and DA were assayed on single whole brain or spinal cord and the number of samples per time point were 4 samples for each time point except for 45 days where three animals were available for the determinations. The results are expressed as % of control.

Uptake of ^3H 5-HT and ^3H -NA was measured *in vitro* on thin circular

slices (3 mm in diameter and 0.5–1.0 mm thick) prepared from cortex, hypothalamus and thoracic segments of the spinal cord according to Hamburger (1967) and Sachs and Jonsson (1972). The slices were incubated as described by Björklund, Nobin and Stenevi (1973 a) the procedure being as follows. After preincubation for 10 min at +37°C in a Krebs-Ringer bicarbonate buffer (pH 7.4 saturated with 95 % O₂ and 5 % CO₂ and containing 1.8 g/l glucose) ³H 5-HT (³H 5-HT creatinine sulphate, 6.8 Ci/mmol gen. labelled Radiochemical Centre, Amersham, England) or ³H NA (L NA 7 ³H, 6.4 Ci/mmol New England Nuclear Frankfurt, G.F.R.) was added to a final concentration of 0.5 × 10⁻⁷ M (³H 5-HT) or 10⁻⁷ M (³H NA). The incubation was continued for a further 10 min and then terminated by the addition of 5 ml ice-cold buffer to each incubation flask, the slices then being transferred to fresh ice-cold buffer. To obtain values for uptake at 0°C, parts of the slices from each region and each control and experimental animal were incubated as above, but the temperature was kept at 0°C. In the ³H 5-HT uptake measurements, both total tritium and unchanged ³H-5-HT were measured according to the procedure described by Björklund, Nobin and Stenevi (1973 a). The values for unchanged ³H 5-HT have been corrected for recovery which was about 45 %. In the ³H NA uptake measurements, the slices were washed for 5 min in fresh buffer at +37°C and then dissolved in 0.5 ml Soluene 100 (Packard Instr. Co.) and subjected to liquid scintillation counting. It has previously been established that unchanged NA constitutes between 85 and 100 % of the total radioactivity measured in this way (Björklund, Nobin and Stenevi 1973 a).

In the scintillation counting the counting efficiency was calculated by means of internal standards.

Fluorescence histochemistry of intraneuronal IA and CA was performed according to the Falck-Hillarp method (for details on the technique, see Björklund, Falck and Owman 1972). Control and 5.7 DHT treated specimens were processed in parallel. Some of the animals used for fluorescence histochemistry were treated with the MAO-inhibitor nialamide (300–500 mg/kg 4–6 hrs before killing). The material used for fluorescence histochemistry comprised rats treated with low (10–75 µg) or high (100–200 µg) doses of 5.7 DHT and included several time points between 45 min and 16 days after injection.

RESULTS

5.7 DHT was administered in doses of 10, 25, 50, 75, 100 or 200 µg (calculated as the base) into the lateral ventricle. The lower doses were well tolerated, whereas the higher ones induced severe convulsions within a few minutes after the injection. These convulsions were totally prevented by the injection of nembutal (sodium pentobarbitone 40 mg/kg i.p.) shortly after the drug administration. With this procedure, which was regularly used with doses of 75 µg 5.7 DHT or higher, almost all animals survived the first month.

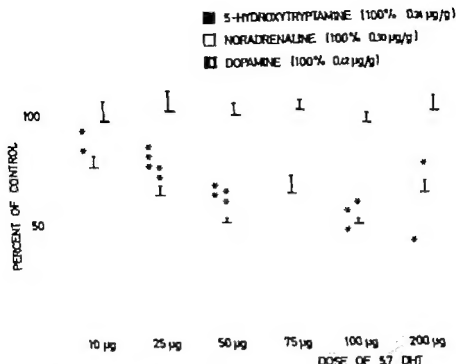


Fig. 1 Effect of six different doses of 5,7 DHT on whole brain 5-HT, NA and DA concentrations, 10 days after intraventricular injection. The bars give means \pm S.E.M. of four determinations. Differences from control values: $=0.05 > p > 0.01$; $=0.01 > p > 0.001$; *** = $p < 0.001$ Student's t-test.

Some of the animals treated with 200 µg 5,7 DHT were allowed to survive longer and within this group about half of the animals died unexpectedly and the remaining ones were therefore killed after 45 days.

As after treatment with 5,6-DHT the animals lost weight (about 10–15 %) after the higher doses of 5,7 DHT. The injected animals exhibited bizarre social behavior and were hyperexcitable (see Baumgarten and Lachenmayer 1972) this condition lasted until they were killed.

Effects of 5,7 DHT on brain and spinal cord monoamine content

Previous investigations on the effects of 5,6-DHT on CNS serotonin neurons showed minimum levels of 5-HT in several CNS regions at 10 days after its intraventricular injection (Baumgarten *et al.* 1971). This time was therefore chosen for an evaluation of the dose response effects of 5,7 DHT on the monoamine concentrations in whole brain (5-HT, NA and DA) and spinal cord (5-HT and NA). Fig. 1 shows that, at 10 days, there was a significant drop in both 5-HT and NA levels in whole brain, even with the lowest amount of 5,7 DHT injected (10 µg free base). With increasing doses the brain NA and 5-HT levels were equally reduced up to 50 µg, a dose that resulted in about 50 % depletion of both amines. At higher doses of 5,7 DHT 75–200

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■ 5-HYDROXYTRYPTAMINE (100% 0.21 µg/g)
 □ NORADRENALINE (100% 0.20 µg/g)
 ▣ DOPAMINE (100% 0.22 µg/g)

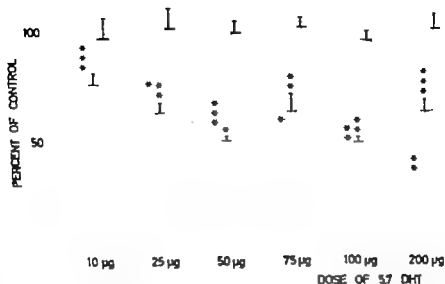


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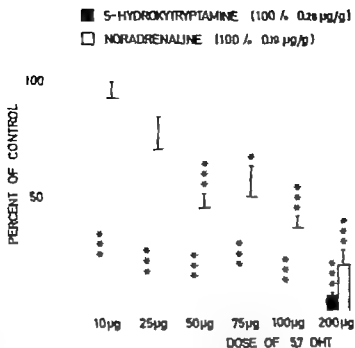


Fig. 2 Effect of six different doses of 5,7 DHT on spinal cord 5-HT and NA concentrations, 10 days after intraventricular injection. The bars give means \pm S.E.M. of four determinations. Differences from control values $\approx 0.05 > p > 0.01$ $\approx 0.01 > p > 0.001$ $\approx p < 0.001$ Student's *t* test.

μ g the 5-HT concentration was further reduced (up to 75 % depletion after 200 μ g) whereas no further decrease was registered in case of NA. In sharp contrast to the marked effect of 5,7 DHT on the NA concentration, the brain DA concentration was unaffected at 10 days after all doses tested (Fig. 1)

In the spinal cord (Fig. 2) a near maximal depletion of serotonin (about 85 % reduction) occurred already after 10 μ g 5,7 DHT. After this lowest dose there was no significant effect on the NA concentration. However with increasing dose of 5,7 DHT the NA concentration of the spinal cord was progressively reduced. After 200 μ g the NA concentration was approximately 15 % of control, the reading values was, however, at the limit of detectability (Fig. 2). At all doses tested spinal cord 5-HT concentrations were more reduced than those of NA. As 10 μ g 5,7 DHT provoked an almost maximal depletion of serotonin in the spinal cord but no significant changes in the NA concentration the specificity of action of 5,7 DHT on serotonin neurons in the spinal cord was greatest at this low dose.

That the percentage reduction of 5-HT was not the same in all brain regions is seen in Figs. 3 A–D. At 10 days after 200 μ g 5,7 DHT the reduction in 5-HT varied from about 55 % in the pons + medulla oblongata preparation to about 85 % in the forebrain rest preparation and the septum. The depletion (at 10 days) was higher in the ventricle near brain regions (septum, striatum)

hypothalamus) and lower in the regions comprising most of the 5-HT cell bodies (mesencephalon and pons+medulla oblongata)

The time course of effects of a single high dose of 5.7 DHT (200 μ g) on CNS serotonin concentrations was evaluated on individual brain regions and on the spinal cord, and the results are summarized in Figs. 1 A—D. Similar to what has been found after 5.6-DHT treatment (Baumgarten *et al* 1971) different patterns of depletion and recovery of serotonin were registered in different CNS regions. In the spinal cord (Fig. 3 A) the striatum (Fig. 3 C) and the forebrain rest preparation (Fig. 3 D) an initial rapid depletion of about 35—60 % of the 5-HT concentration seen at 3 hrs after drug injection, was followed by a more protracted and gradual decline giving minimum levels (10—25 % of control) at 10 days. The concentrations then remained unchanged for at least another 20 days. A slight, statistically not significant recovery was noticed in both spinal cord and the striatum between 1 month and 45 days. In remaining brain regions — septum, hypothalamus, mesencephalon and pons+medulla oblongata — the acute 5.7 DHT depletion, registered at 3 hrs or 1 day was more pronounced (between 60 and 80 %) within the subsequent 3 or 4 days, there was a variable, partial, recovery of the 5-HT concentrations. This recovery was most pronounced in mesencephalon and pons+medulla oblongata, which are the regions rich in serotonin-containing cell bodies — in these regions the recoveries between 3 hrs and 4 days were highly significant ($p < 0.001$). In pons+medulla oblongata, mesencephalon, hypothalamus and septum there was, in addition, a tendency to a late slow recovery of the 5-HT concentrations between 10 and 45 days.

Whereas the brain DA levels were only little affected at all time points up to 10 days (Fig. 4) 200 μ g of 5.7 DHT caused a strong and rapid depletion of NA in brain (Fig. 4) and in spinal cord (Fig. 5) and at 3 hrs after injection the NA concentrations were about 25 % of control. By 4 days after injection the NA levels had recovered partially being 45 % of control in the brain and 70 % of control in the spinal cord. These recoveries between 3 hrs and 4 days were statistically significant ($0.001 < p < 0.01$ in both brain and spinal cord). Interestingly in the spinal cord (but not in the brain) there was a second phase of NA depletion between 4 and 10 days, from about 70 % of control at 4 days to about 15 % at 10 days (difference between 4-day and 10-day values $p < 0.001$).

The reduction in brain and spinal cord NA remained up to the 45th day. In fact, there was a tendency for a further reduction in brain NA concentration between 10 and 45 days (difference between 10-day and 45-day values $0.01 < p < 0.05$). In contrast, the whole brain DA concentration was increased at 45 days (135 % of control) — this increase was not statistically significant, however.

Effects of 5.7 DHT on 3 H-5-HT and 3 H NA uptake by slices of brain and spinal cord in vitro

The effect of one injection of 200 μ g 5.7 DHT on the capacity of thin slices

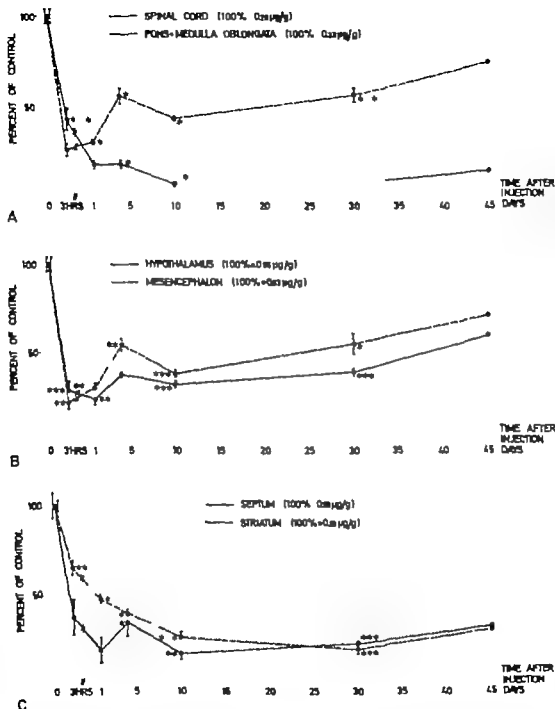
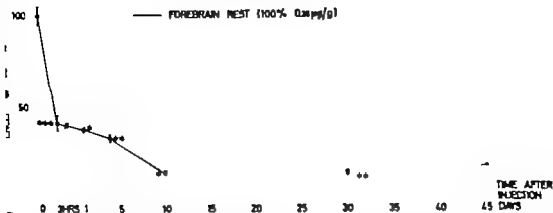


Fig. 3 A—D Time course of effect of one single intraventricular injection of 200 µg 5,7 DHT on the 5-HT concentration in seven brain regions. Preparations from four or five rats were pooled for each determination. Each value except the 45-day values is



the mean \pm S.E.M. of four to eight determinations. The 45-day values are the mean of two determinations. Differences from control values. $=0.05 > p > 0.01$
 $**=0.01 > p > 0.001$ $***=p < 0.001$ Student's *t* test.

from cortex, hypothalamus and spinal cord to accumulate tritiated 5-HT or NA during a short (10 min) incubation in vitro was studied at 9–11 days after injection (Fig. 6). The 5.7 DHT treatment caused a strong and statistically highly significant reduction in ^3H 5-HT and ^3H NA uptake in all three regions. Regarding the ^3H 5-HT uptake, the reduction was seen both in the measurements of unmetabolized 5-HT (hatched bars in Fig. 6) and in the measurements of total radioactivity corresponding to 5-HT plus metabolites (open bars in Fig. 6). The reduction in the uptake of unmetabolized ^3H 5-HT was between 85 and 90% in all three regions, whereas the reduction in ^3H NA uptake was less (40–57%). These effects on the ^3H 5-HT and ^3H -NA uptake are quite comparable to the reductions registered in the levels of endogenous 5-HT and NA at 10 days after 200 μg 5.7 DHT (see above, Figs. 3, 4 and 5).

The percentage unmetabolized ^3H 5-HT of the total accumulated tritium in the slices was less (about 15–25%) in the 5.7 DHT treated animals than in the control animals (about 40–55%). This signifies that, due to the strong inhibition of the neuronal uptake, the accumulated 5-HT was more rapidly metabolized in the 5.7 DHT treated rats. As a consequence, the reduction in the uptake of unmetabolized ^3H -5-HT was greater than that recorded in the total accumulated tritium (Fig. 6).

Preliminary fluorescence histochemical observations in 5.7 DHT-treated rats

Observations were made in rats after survival times varying from 45 min to 16 days, some of the long term animals being treated with the MAO-inhibitor nialamide (300–500 mg/kg 4–6 hrs before killing) to facilitate the visualization of IA-containing terminals.

At 45 min after the 5.7 DHT injection a diffuse, bright yellow-green formaldehyde induced fluorescence occurred in the periventricular as well

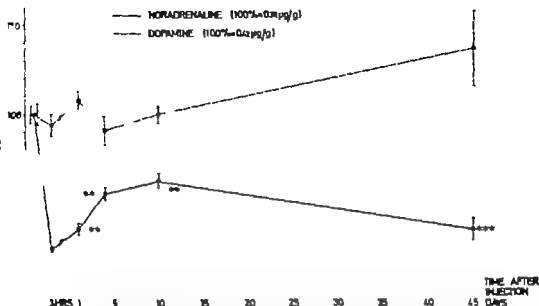


Fig 4 Time course of effect of one single intraventricular injection of 200 µg 5,7 DHT on whole brain NA and DA concentrations. Each point gives the mean \pm S.E.M. of five determinations. Differences from control values $^{**}=0.05 > p > 0.01$ $^{***}=0.01 > p > 0.001$ $^{****}=p < 0.001$ Student's t test.

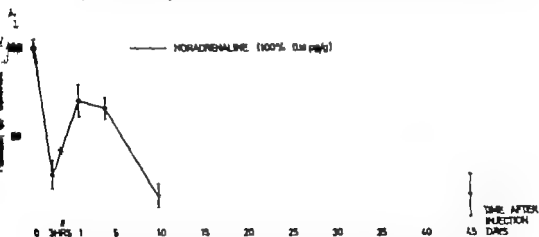


Fig 5 Time course of effect of one single intraventricular injection of 200 µg 5,7 DHT on spinal cord NA concentration. Each point gives the mean \pm S.E.M. of three to five determinations. Differences from control values $^{**}=0.05 > p > 0.01$ $^{***}=0.01 > p > 0.001$ $^{****}=p < 0.001$ Student's t test.

as in other surface-bordering parts of the brain. The colour of this fluorescence was clearly different from that of the CA or the 5-HT fluorophores, but similar to that of the fluorophore of authentic 5,7 DHT in models (unpublished observations). The yellow-green fluorescence — thus probably identical to the injected 5,7 DHT — was partly concentrated in structures resembling vari-

- ☐ ^3H -5-HT UPTAKE, TOTAL TRITIUM
☒ ^3H -5-HT UPTAKE, UNCHANGED 5-HT
☒ ^3H NA UPTAKE, TOTAL TRITIUM

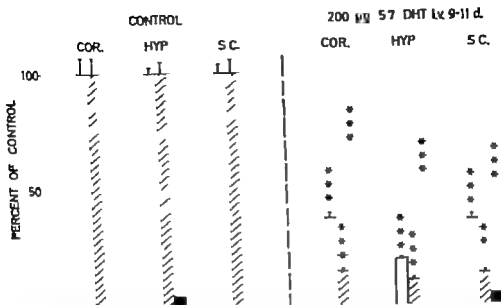


Fig. 6 Effect of one injection of 200 µg of 57 DHT on (H)5-HT and (^3H)NA uptake by brain and spinal cord slices in vitro, 9–11 days after injection. Slices were obtained from cortex (COR.) hypothalamus (HYP) and spinal cord (S.C.) and the values are expressed as percent of untreated controls, and are given as means \pm S.E.M. of 6–15 determinations. The values represent active uptake calculated as the uptake measured at +37°C minus the uptake measured at 0°C. Differences from control values: * = 0.05 > p > 0.01 ** = 0.01 > p > 0.001 *** = p < 0.001 Student's t-test.

some monoamine axons and axon terminals. Some of these fibres — e.g. in the suprachiasmatic nucleus, the subcommissural organ and the neuropil underneath the ependyma of the fourth ventricle — were probably identical to IA-containing fibres, whereas others — e.g. in the periventricular hypothalamus and septum — could represent NA-containing ones. In addition, fluorescence occurred in varicose fibres within the medial forebrain bundle and in the medulla oblongata, close to the ventral surface, probably indicating an uptake of 57 DHT into the ascending and descending non-terminal monoamine axon bundles.

The 57 DHT induced yellow-green fluorescence was observable up to at most, 8–12 hrs after injection thereafter the yellow fluorescent IA-containing fibres that normally are present in ventricle near regions (e.g. the suprachiasmatic nuclei and the subcommissural organ) could no longer be demonstrated. Higher doses of 57 DHT (100–200 µg) caused a rapid disappearance of yellow fluorescent IA-containing fibres from ventricle near regions and partly also from ventricle-distant structures, such as the globus pallidus, the

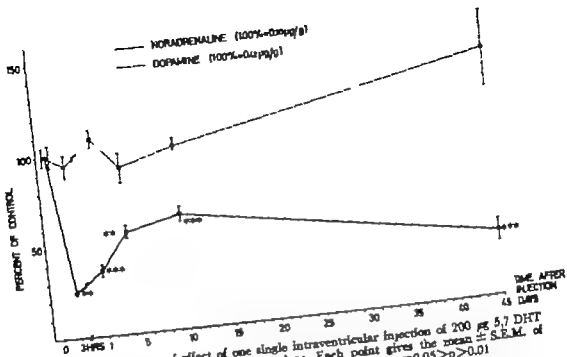


Fig 4 Time course of effect of one single intraventricular injection of 200 µg 5,7 DHT on whole brain NA and DA concentrations. Each point gives the mean \pm S.E.M. of three to five determinations. Differences from control values: $=0.05 > p > 0.01$ $*** = p < 0.001$ Student's t-test.

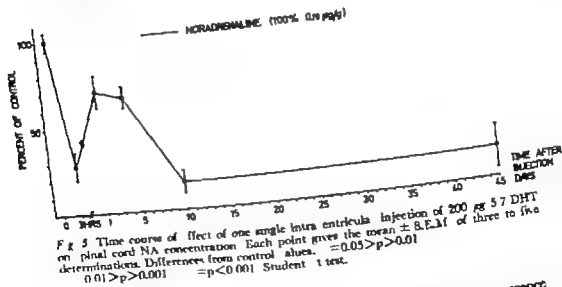


Fig 5 Time course of effect of one single intra-ventricular injection of 200 µg 5,7 DHT on spinal cord NA concentration. Each point gives the mean \pm S.E.M. of three to five determinations. Differences from control values: $=0.05 > p > 0.01$ $*** = p < 0.001$ Student's t-test.

in other surface-bordering part of the brain. The colour of this fluorescence was clearly different from that of the CA or the 5-HT fluorophores, but was like to that of the fluorophore of authentic 5,7 DHT in models (unpublished observations). The yellow-green fluorescence — it is probably identical to the injected 5,7 DHT — was partly concentrated in structures resembling var-

In contrast to 5,6-DHT 5,7-DHT had pronounced and persisting effects also on the central NA neurons. Thus, 200 μ g 5,7-DHT caused a 40–85 % reduction in brain and spinal cord NA levels that persisted for at least 45 days, and there was a 40–57 % reduction in the ^3H NA uptake capacity of slices from cortex, hypothalamus and spinal cord at 9–11 days after injection, suggesting that 5,7-DHT induces degeneration also in NA neurons. This is further supported by the fluorescence microscopical observations of a long term disappearance of fluorescent, presumed NA terminals, and of signs of extensive axonal damage in NA axon bundles. It is notable that, following doses up to 50 μ g the degree of depletion at 10 days was similar for 5-HT and NA in the brain. After higher doses (75–200 μ g) 5-HT was further reduced (up to 75 % after 200 μ g) whereas NA remained at about 50 % of control. It is remarkable that even after 200 μ g of 5,7-DHT the DA neurons appeared largely unaffected by the treatment.

Studies on the impairment of ^3H 5-HT and ^3H NA uptake into cortical slices *in vitro* induced by a preceding exposure of the slices to 5,7-DHT (Björklund, Baumgarten, Nobin and Schönlager in preparation) have provided evidence that 5,7-DHT exerts direct toxic effects on both IA and CA axons, but that the effect on the IA axons is considerably stronger. This has also been shown in studies on the effects of localized, intracerebral or intraspinal injections of 5,7-DHT (Björklund, Nobin and Stenevi 1973 c) demonstrating that IA axons are more susceptible than CA axons to 5,7-DHT but that both DA and NA fibres are partially damaged. The fact that after intraventricular injections this higher susceptibility of the IA neurons to 5,7-DHT is revealed in the brain only after the highest doses is most probably due to the difference in topography of the different monoamine neuron systems, and this readily explains also why the IA neurons innervating the spinal cord are much more susceptible than those of the brain to neurotoxic drugs administered via the cerebrospinal fluid (see Nobin *et al.* 1973). In the brain, extensive NA containing terminal systems are located close to the ventricles and one of the major ascending NA fibre bundles (the so-called dorsal CA bundle) is situated close to the aqueduct (see Ungerstedt 1971). This is in contrast to the IA containing fibre systems which are more evenly distributed in the brain. In spinal cord the situation is rather the reverse: here, the descending IA axons are located very superficially in the white matter and are thus easily reached from the cerebrospinal fluid (Nobin *et al.* 1973).

At the same dose level, e.g. 50 or 75 μ g, 5,6-DHT and 5,7-DHT have roughly the same depletory effect on the whole brain or spinal cord 5-HT concentrations (compare with data from Baumgarten *et al.* 1971, Da Prada, Carruba, O'Brien, Saner and Pletscher 1972, Baumgarten, Björklund and Nobin, unpublished observations). This is interesting in view of the uptake affinity data of Horn *et al.* (1973). According to their measurements, the affinity of 5,7-DHT for the 5-HT uptake mechanism (measured as the concentration of the drug causing 50 % inhibition of the uptake and retention of ^3H 5-HT by synaptosomes from rat hypothalamus) was about 6 times less than

that of 5,6-DHT. Thus, while 5,6-DHT has a very high affinity for the 5-HT uptake mechanism (comparable to 5-HT itself) and a much lower affinity for the NA or DA uptake mechanisms (Baumgarten *et al.* 1972 b) 5,7-DHT has, in fact, a higher affinity for the NA uptake mechanism than for the 5-HT mechanism (Horn *et al.* 1973). Since the selective neurotoxic action of the dihydroxylated tryptamines on the monoamine neurons most probably depends on their uptake and accumulation within the neurons, it is notable that 5,7-DHT is as potent on the serotonin neurons as 5,6-DHT despite its considerably lower affinity for the 5-HT uptake system. This points to a higher general neurotoxic potency of 5,7-DHT which at least partly could be due to 5,7-DHT not being as sensitive to oxidation at physiological pH as 5,6-DHT which to some extent precipitates in the form of a brownish pigment on the ventricle walls. The higher stability of 5,7-DHT will most likely provide the substance with better diffusion properties and allow it to reach deeper into the brain tissue.

Although the neurotoxic effects of 5,7-DHT and 5,6-DHT with respect to the serotonin neurons appears to be similar 5,7-DHT has a clear advantage over 5,6-DHT in being markedly less toxic. This allowed the administration of much higher doses of 5,7-DHT into the cerebrospinal fluid and made possible a more pronounced denervation of several brain regions. With 5,6-DHT a comparable degree of denervation can be achieved in diencephalic and telencephalic areas only after local injections close to the ascending LA fibre systems (Björklund *et al.* 1973 c). On the other hand, the selectivity of 5,7-DHT is less, and it is clear that 5,7-DHT affects the NA neurons to a considerably greater extent than 5,6-DHT does.

Two different patterns could be distinguished in the timecourse of effects of 5,7-DHT on the 5-HT content in different CNS regions. (1) In ventricle near regions (septum and hypothalamus) as well as in the cell body rich parts (mesencephalon and pons+medulla oblongata) a profound reduction in 5-HT (up to 80 %) was registered by 3–24 hrs. Following a transient recovery phase between 1 and 4 days after injection, the 5-HT levels declined to near minimum values (55–85 % reduction) at 10 days. (2) The regions comprising tissue more distant from the ventricles — the spinal cord, the forebrain rest sample and the striatum — an initial, rapid depletion of about 35–60 % of the 5-HT concentration, seen at 3 hrs after injection, was followed by a more protracted and gradual decline giving minimum levels (10–25 % of control) at 10 days. The patterns of 5-HT depletion should be possible to interpret as a sequence of different events that occur to a different extent in different regions, and that cannot be clearly separated from one another: (a) an initial rapid displacement phase, followed by (b) a recovery of some of the depleted terminals, and by (c) an acute degeneration of other terminals. These events are followed by (d) a protracted phase of anterograde degeneration of terminals that were not initially reached by the drug.

The several events induced by 5,7-DHT in the central serotonin neurons are similar to those previously described in detail by Baumgarten *et al.* (1971).

Nobin *et al.* (1973) and Björklund *et al.* (1973 a, b c) after 56-DHT treatment. However because 57 DHT was administered in higher doses than 56-DHT the magnitude of both the acute (3—24 hrs after injection) and the long term effects was greater after 57 DHT (200 µg) than after 56-DHT (75 µg)

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Supplementum 392

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BY

SIVERT LINDSTRÖM

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This thesis mainly constitutes a summary of the following articles:

- I Hultborn H E Jankowska and S Lindström Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurons J Physiol (Lond) 1971 15 591-61
- II Gustafsson B and S Lindström Recurrent control from motor axon collaterals of Ia inhibitory pathways to ventral spinocerebellar tract neurones Acta physiol scand 1973 In press
- III Lindström S and E D Schomburg Group I inhibition in Ib excited ventral spinocerebellar tract neurones Acta physiol scand 1973 In press
- IV Lindström S and E D Schomburg Recurrent inhibition from motor axon collaterals of ventral spinocerebellar tract neurones Acta physiol scand 1973 In press

The papers are referred to by their Roman numerals in the text

INTRODUCTION

The inhibitory pathways from large muscle spindle (Ia) afferents to motoneurons and from motor axon collaterals to motoneurons are two of the best known inhibitory pathways in the mammalian central nervous system. The former was described by Lloyd (1941 1943 a b 1946 a, b) who found that the largest muscle afferents which supply monosynaptic excitation to motoneurons innervating their own and synergistic muscles also give reciprocal inhibition to motoneurons of muscles acting as antagonists at the same joint. Lloyd (1943 b c) further showed that the excitatory two-neurone reflex arch from these afferents formed the basis for the stretch reflex of Liddell and Sherrington (1924). Extensor and flexor muscles acting at a given joint are thus linked by the excitatory and inhibitory reflex connecting from these afferents into a 'myotatic unit' (Lloyd 1946 b). Already these early studies indicated that the afferents responsible for the effects originate from muscle spindle primary endings, a view which has been fully substantiated by later experiments (cf. Matthews 1972).

The reciprocal Ia inhibitory pathway was considered by Lloyd to be monosynaptic like the Ia excitatory path - thus the name direct inhibition. However, later experiments have demonstrated that an interneurone is interposed in the inhibitory pathway. This conclusion rests on precise measurements of the latencies of the inhibitory responses in motoneurons (Eccles, Fatt and Lundgren 1956; Araki, Eccles and Ito 1960) and on the demonstration of spatial facilitation in the pathway (R. M. Eccles and Lundberg 1968 a). The interposed interneurone was initially seen largely as a device to change the excitatory action of primary afferents into inhibition (cf. Eccles *et al.*, 1956) but later investigators have stressed the integrative role of this interneurone (cf. Lundberg 1970; Hultborn 1972 a, b). Several descending and segmental systems have been found to converge onto the Ia inhibitory interneurons (cf. Lundberg 1970; Hultborn 1972 a, b) and the first part of this study added a further one, namely the recurrent inhibitory path from motor axon collaterals through Renshaw cells.

That impulses in motor axons can affect the excitability of other motoneurons was demonstrated by Renshaw (1941) who activated motor axons antidromically and observed that such stimuli caused inhibition or facilitation of other motoneurons. The recurrent inhibitory pathway to motoneurons was subsequently analyzed in detail by Eccles, Fatt and Koketsu (1964) who gave convincing evidence for a disynaptic action from motor axon collaterals, through a specific

inhibitory interneurons. This interneuron was named the Renshaw cell by Eccles *et al* (1954) in honor of Renshaw who first described these cells (Renshaw 1946).

The recurrent facilitatory effects have been analyzed by Wilson and collaborators (Wilson 1959, Wilson, Diecke and Talbot 1960, Wilson and Burgess 1962 a, b). They showed that the facilitation is in fact a disinhibition, due to recurrent inhibition from motor axon collaterals through Renshaw cells of some inhibitory interneurons tonically impinging upon motoneurons. This finding formed the starting point for the first part of the present study. On the assumption that these recurrently inhibited interneurons were interposed as a link in some inhibitory pathway to motoneurons and that the recurrent control of any such pathway was functionally important, the effect of stimulation of motor axon collaterals was tested on transmission in different segmental inhibitory reflex pathways to motoneurons. As mentioned, the interneurons in the reciprocal Ia inhibitory pathway to motoneurons were found to receive recurrent inhibition from motor axon collaterals. This finding has initiated a series of studies which have considerably increased our knowledge about the Ia inhibitory pathway both from a physiological and anatomical point of view (Hultborn, Jankowska and Lindström 1971 a, b, Hultborn, Jankowska, Lindström and Roberts 1971, Fedina and Hultborn 1972, Hultborn 1972 a, b, Hultborn and Lundberg 1972, Hultborn and Santini 1972, Hultborn and Udo 1972 a, b, c, Jankowska and Roberts 1972 a, b, Jankowska and Lindström 1972, Jankowska, Lundberg and Stuart 1973).

Disynaptic inhibition from Ia afferents is evoked not only in motoneurons but also in cells of origin of the ventral (Eccles, Hubbard and Oscarsson 1961) and the dorsal spinocerebellar tracts (Curtis, Eccles and Lundberg 1958, Eccles, Oscarsson and Willis 1961). Parallel with the above studies of the recurrent control of the reciprocal Ia inhibitory pathway to motoneurons, Lundberg and Weight (1971) performed experiments on the ventral spinocerebellar tract (VSCT) which led to the hypothesis that the VSCT conveys information about transmission in segmental inhibitory pathways to motoneurons (Lundberg 1971). This hypothesis should be considered in relation to the extensive convergence from different segmental and descending sources onto the interneurons in such reflex pathways (cf. Lundberg 1966, 1969 a, 1970, Hultborn 1972 b and Fig. 1) suggesting that higher centres might need some feedback information from these interneurons in order to achieve an accurate control of movements.

Central to the new VSCT hypothesis is the postulate that VSCT neurons receive collateral connections from the last order inhibitory interneurons in reflex pathways to motoneurons (Lundberg 1971). The recurrent control of the interneurons in the reciprocal Ia inhibitory

pathway to motoneurons offered an excellent opportunity to test this postulate. Accordingly the observed parallelism in the recurrent control of Ia inhibitory pathways to motoneurons and VSCT neurons provides strong indirect evidence that these two systems do receive collateral connections from common interneurons. The second part of this thesis is devoted to an evaluation of the capability of the VSCT to signal information about inhibitory pathways to motoneurons using the reciprocal Ia inhibitory pathway as a model.

Inhibitory interneurons This interneurone was named the Renshaw cell by Eccles *et al* (1964) in honor of Renshaw who first described these cells (Renshaw 1946)

The recurrent facilitatory effects have been analyzed by Wilson and collaborators (Wilson 1959 Wilson Diecke and Talbot 1960 Wilson and Burgess 1962 a b) They showed that the facilitation is in fact a disinhibition due to recurrent inhibition from motor axon collaterals through Renshaw cells of some inhibitory interneurons tonically impinging upon motoneurons This finding formed the starting point for the first part of the present study On the assumption that these recurrently inhibited interneurons were interposed as a link in some inhibitory pathway to motoneurons and that the recurrent control of any such pathway was functionally important, the effect of stimulation of motor axon collaterals was tested on transmission in different segmental inhibitory reflex pathways to motoneurons As mentioned, the interneurons in the reciprocal Ia inhibitory pathway to motoneurons were found to receive recurrent inhibition from motor axon collaterals This finding has initiated a series of studies which have considerably increased our knowledge about the Ia inhibitory pathway both from a physiological and anatomical point of view (Hultborn Jankowska and Lindström 1971 a b Hultborn, Jankowska Lindström and Roberts 1971 Fedina and Hultborn 1972 Hultborn 1972 a b Hultborn and Lundberg 1972 Hultborn and Santini 1972 Hultborn and Udo 1973 a, b Jankowska and Roberts 1972 a, b Jankowska and Lindström 1972 Jankowska Lundberg and Stuart 1973)

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Central to the new VBCT hypothesis is the postulate that VBCT neurones receive collateral connexions from the last order inhibitory interneurons in reflex pathways to motoneurons (Lundberg 1971) The recurrent control of the interneurons in the reciprocal Ia inhibitory

volleys do not elicit changes in the membrane conductance of the recorded cells or primary afferent depolarization this method allows conclusions to be drawn regarding the convergence on the interneurons in the studied reflex pathway. An increase of test IPSPs by the conditioning volleys indicates an excitatory convergence onto the interneurons and a decrease an inhibitory one. This indirect approach is superior to direct recordings from interneurons as a first step in analysing interneuronal properties since individual interneurons usually cannot be identified as belonging to a defined reflex pathway (cf. Lundberg 1969 a)

Electrical stimulation of peripheral nerves and ventral roots has been used throughout this study since this method allows accurate timing of the responses and controlled activation of afferents in several muscle and cutaneous nerves in the same experiment. Single stimuli were used both for evoking the test responses from different types of afferents and for the conditioning stimulation of ventral roots. The test IPSPs were usually submaximal for a given fibre group in order to increase the sensitivity of the testing while the conditioning ventral root volleys were maximal for α -fibres. In the motoneurone experiments (paper I) only those of the ventral roots L5 - S1 which did not evoke recurrent inhibition in the recorded motoneurons were used for conditioning stimulation while all the ventral roots L5 - S1 were used in the VSCT experiments (papers II and III) since the recorded VSCT neurones did not receive recurrent inhibition from motor axons (cf. however paper IV). The unconditioned and the conditioned responses were usually averaged to minimize errors due to synaptic noise and random variations in the responses.

The analysis has been concerned mainly with synaptic effects from group I muscle afferents but group II and III muscle afferents and cutaneous afferents have also been stimulated. The group I muscle afferents are connected with muscle spindle primary endings (Ia afferents) and with Golgi tendon organs (Ib afferents). The difference in threshold for electrical stimulation of these afferents has been utilized to differentiate between the synaptic responses evoked by them. The main conclusions rest on observations of effects from nerves to thigh muscles for which the electrically elicited group I nerve volleys usually display a double configuration in triphasic recordings from the dorsal root entry zone (Bradley and Eccles 1953). It is well established that the first low threshold component in such group I volleys is mainly due to activation of Ia afferents and that the second higher threshold component largely is due to activation of Ib afferents (Bradley and Eccles 1953, Laporte and Beeson 1957, Eccles *et al* 1957 a & c cf. also Matthews 1972). Even if a certain degree of overlap exists between these two groups of afferents the main contribution to the recorded synaptic potentials from Ia or Ib

afferents can usually be determined by carefully grading the responses by varying the strength of stimulation. The differentiation is considerably sharpened up by testing with the double volley technique (Bradley and Eccles 1953; Eccles, Eccles and Lundberg 1957 a) which usually has been used in this study. By careful grading it is often also possible to classify tentatively the effects from nerves which do not display clearcut differences in the conduction velocity of the Ia and Ib fibres (usually nerves to leg muscles) into Ia or Ib responses, since also in such cases the Ia fibres are largely recruited in the lower threshold group I range and the Ib fibres in the higher threshold group I range (Eccles *et al* 1957 a & c; Matthews 1972). The effects from high threshold muscle afferents (group II and III) and cutaneous afferents were confined to the flexor reflex pattern (FRA; R. M. Eccles and Lundberg 1950; Holmqvist and Lundberg 1961) and were usually easy to differentiate from responses of group I afferents.

Although synchronous electrical stimulation of peripheral nerves is a highly unnatural way of activating afferents from different types of receptors this method has proved to be a valuable tool in determining central actions of such afferents. To understand the functional significance of connections found in this way it is necessary to know when and to which degree they are utilized in transmission of adequately evoked discharges, an information which cannot be obtained with the present technique. However the results offer a useful basis for tentative interpretations which subsequently can be tested with other experimental approaches.

ABBREVIATIONS

DSCT: dorsal spinocerebellar tract; VSCT: ventral spinocerebellar tract; EPSP: excitatory postsynaptic potential; IPSP: inhibitory postsynaptic potential; FRA: flexor reflex afferents

RESULTS AND COMMENTS

A Recurrent effects from motor axon collaterals on interneurons in Ia inhibitory pathways

1 To α -motoneurons Disynaptic IPSPs are evoked in α -motoneurons largely from Ia afferents in nerves to antagonist muscles (Lloyd 1946 b; R. M. Eccles and Lundberg 1956 b). In paper I it was demonstrated that such IPSPs are effectively depressed by a preceding conditioning antidromic volley in motor axons. The effect was found on Ia IPSPs in practically all tested motoneurons supplying different flexor or extensor muscles of the hindlimb and seems therefore to be a general property of Ia inhibitory pathways to hindlimb motoneurons. Since the depression occurred without any concomitant conductance change in the membrane of the recorded motoneurons and without excitability changes in Ia primary afferents, it was concluded that the observed effect was due to inhibitory interaction at the interneuronal level in the Ia inhibitory pathway.

By varying the interval between the conditioning and the testing volleys it was found that the onset of the depression occurred at an interval indicating a disynaptic linkage from motor axons to the Ia inhibitory interneurons and that the time course of the depression corresponded to the time course of recurrent inhibition of motoneurons (Renshaw 1941; Eccles *et al.* 1954). This and some other observations led to the conclusion that the recurrent depression of the Ia IPSPs was caused by postsynaptic inhibition of the Ia inhibitory interneurons mediated from motor axon collaterals through Renshaw cells. The pathway should thus be similarly organized as the recurrent inhibitory pathway to α -motoneurons (Eccles *et al.* 1954).

The above conclusions have been fully supported in subsequent investigations which will be briefly summarized. By means of mono-synaptic test reflexes (cf. Fig. 8, paper I) it has been found that the duration of the recurrent depression of the Ia inhibition of α -motoneurons is prolonged by intravenous administration of the anticholinesterase, eserine and decreased by the cholinergic blocking agent, dihydro- β -erythroidine (Gustafsson and Lindström, unpublished). This indicates that the recurrent inhibitory pathway to the Ia inhibitory interneurons, like the one to motoneurons, contains a cholinergic synapse (between motor axon collaterals and Renshaw cells; cf. Eccles *et al.* 1954).

Interneurons which fulfil all requirements of cells which should relay reciprocal Ia inhibition to motoneurons e.g. monosynaptic excitation from Ia afferents and disynaptic inhibition from motor axons have been found in the ventral horn just dorsomedially of the motor nuclei (Hultborn et al 1971 a). By simultaneous recording from such Ia interneurons and supposed target motoneurons Jankowska and Roberts (1972 b) were able to show that action potentials in the interneurons were followed by monosynaptic unit IPSPs in the motoneurons thereby obtaining direct proof that these interneurons were interposed as a link in the reciprocal Ia inhibitory pathway to motoneurons. The disynaptic Ia inhibitory pathway to motoneurons is thus the first interneuronal pathway in the mammalian central nervous system which has been fully identified physiologically in all parts. The interneurons in this pathway have been identified also morphologically by intracellular staining with Procion Yellow (Jankowska and Lindström 1972, 1973). It should be pointed out that these interneurons are not identical with the Ia excited interneurons in the intermediate region which originally were supposed to mediate Ia reciprocal inhibition to motoneurons (Eccles et al 1956). These latter interneurons lack recurrent inhibition and presumably project to some other neurones than motoneurons (Hultborn et al 1971 a cf. Discussion paper III).

The recurrent inhibition of α -motoneurons is supplied by α -motor axon collaterals (Eccles et al 1954) and the same is true for the recurrent depression of Ia IPSPs in motoneurons (paper I). However different motor axons are responsible for the recurrent inhibition of a given motoneuron and for the depression of Ia IPSPs in the same cell.

The recurrent inhibition is supplied mainly by efferents to muscles coupled in Ia synergism while nerves to antagonist muscles are without effects (Eccles, Eccles Iggo and Ito 1961, Hultborn et al 1971 b). Since there is generally an overlap in the rostrocaudal distribution of synergistic motor nuclei the recurrent inhibition is evoked mainly from ventral roots neighbouring the one containing the axon of the recorded cell. This is best illustrated with motoneurons of the antagonist muscles posterior biceps-semitendinosus (knee flexors) and quadriceps (knee extensors) which are located at two different levels in the spinal cord, posterior biceps-semitendinosus motoneurons in the L7 - S1 segments and quadriceps motoneurons in the L5 and rostral L6 segments. The former cells receive their main recurrent inhibition from the L7 and S1 ventral roots and the latter their main recurrent inhibition from the L5 and L6 ventral roots (cf. paper I).

The Ia IPSPs in the posterior biceps-semitendinosus motoneurons (evoked by Ia afferents in the quadriceps nerve) are on the other hand depressed from the L5 and L6 ventral roots but not from the L7 and S1 ventral roots (paper I) while the Ia IPSPs in quadriceps motoneurons

(evoked by Ia afferents in the posterior biceps-semi-tendinosus nerve) are depressed from the L5 and L6 ventral roots but not from the L7 - S1 ventral roots. This finding gave the first indication that the Ia inhibitory interneurons are recurrently inhibited mainly from efferents to muscles which supply the interneurons with Ia excitation and in a subsequent study in which the effects on Ia IPSPs of stimulation of efferents in several different hindlimb nerves were tested, this was indeed found to be the case (Hultborn et al 1971 b). The pattern of convergence of recurrent inhibition onto Ia inhibitory interneurons and α -motoneurons supplied with the same Ia excitation was so similar that Hultborn et al (1971 b) concluded that the same Renshaw cells seem to project to both groups of neurones.

2 To VSCT neurones. The VSCT neurones can be divided into three main groups depending on whether they receive monosynaptic excitation from either Ia or Ib afferents or are without monosynaptic excitation from primary afferents (Oscarsson 1957, Eccles et al 1961 a, Lundberg and Weight 1971). A fourth smaller group of VSCT neurones seems to receive convergence of monosynaptic excitation from both Ia and Ib afferents (Eccles et al 1961 a, Lundberg and Weight 1971 paper III). Disynaptic Ia IPSPs similar to those in motoneurons are evoked in all these categories of VSCT neurones from nerves to hip and knee muscles most frequently from the nerve to the knee extensor quadriceps (Eccles et al 1961 a, Lundberg and Weight 1971 paper II and III). In the majority of the Ia excited VSCT cells and those without group I excitation the Ia IPSPs were susceptible to recurrent depression from motor axon collaterals (paper III) while the Ia IPSPs in a considerable proportion of the Ib cells and in all the Ia/Ib cells were unaffected (paper III). For the following discussion it is important that disynaptic Ia IPSPs are evoked in Ia VSCT neurones either from the same nerve as the one supplying monosynaptic Ia excitation to the cell or from the nerve to the antagonist muscle of the one supplying the Ia excitation (Lundberg and Weight 1971). In both these combinations the Ia IPSPs were susceptible to recurrent depression (paper II).

The recurrent depression of Ia IPSPs in the VSCT neurones occurred without any recurrent inhibitory effect on the VSCT cells themselves (cf paper IV and section D). The latencies and time courses of the depression were similar to those in motoneurons (cf papers I and II). Further the effect was evoked from α -efferents and IPSPs from the quadriceps nerve were depressed from the L5 and L6 ventral roots but not from the L7 and S1 ventral roots while IPSPs from the posterior biceps-semi-tendinosus nerve were depressed from the L7 and S1 ventral roots but not from the L5 and L6 ventral roots (papers II and III). There is thus a striking correspondence between the recurrent effects on Ia IPSPs in VSCT neurones and in motoneurons.

It was carefully controlled that the negative findings with respect to recurrent effects on Ia IPSPs in some of the Ib VSCT neurones were not due to inadequate testing or to failing preparations. In paper III it was therefore concluded that Ia IPSPs are mediated to Ib VSCT neurones by two different groups of Ia inhibitory interneurones with and without recurrent inhibition from motor axon collaterals. The two types of Ia IPSPs had similar thresholds and time courses and were evoked from afferents in the same nerve (quadriceps). The only apparent difference between the Ib cells with the two types of Ia IPSPs was in the pattern of Ib excitation. In cells with the main Ib excitation from the nerves to hip extensors and knee flexors the Ia IPSPs were recurrently depressed while they were unaffected in all the other Ib VSCT cells. At present it is very difficult to interpret the functional significances of these findings (of Discussion paper III). However one important conclusion to be drawn is that seemingly similar postsynaptic potentials evoked in VSCT neurones from the same kind of afferents in the same nerve may have an entirely different function.

3 To DSCT neurones. Disynaptic Ia IPSPs are frequently evoked also in DSCT neurones with monosynaptic excitation from group I muscle afferents (Curtis *et al.*, 1968; Eccles *et al.*, 1961b; Lindström and Takata 1973). The susceptibility of such IPSPs to recurrent depression from motor axon collaterals has been tested and in no case there was any trace of recurrent effects onto them (Lindström and Takata 1973). The Ia inhibition of the DSCT neurones is thus relayed by other interneurones than the Ia inhibition of motoneurones and most VSCT neurones. Although some Ib VSCT cells receive Ia IPSPs which are unaffected by ventral root volleys it is not likely that this effect is mediated by the Ia inhibitory interneurones projecting to the DSCT cells because of the difference in convergence onto the cells. The latter often receive convergence of Ia inhibition from several hindlimb nerves and testing with spatial facilitation has revealed that this convergence occurs already at the inter-neuronal level (Lindström and Takata 1973). The Ia IPSPs in the Ib VSCT neurones on the other hand, are with few exceptions evoked exclusively from the quadriceps nerve. The location of the interneurones which mediate the Ia inhibition to the DSCT neurones is unknown. It is however possible that these cells are located close to the DSCT neurones in Clarke's column, since interneurones with monosynaptic Ia excitation have been found in this region (Lindström and Takata, unpublished).

4 To segmental interneurones. The occurrence of Ia IPSPs in segmental interneurones has not been systematically studied but such effects have been observed in interneurones in the intermediate region of the spinal cord (Hongo, Jankowska and Lundberg 1966, 1972). A small number of such interneurones (all with monosynaptic group I

excitation) have been tested with respect to recurrent Ia IPSPs and in all cases these IPSPs were unaffected by root volleys (Lindström and Schomburg unpublished).

Of special interest for the following discussion is the locally inhibited Ia interneurons in the ventral horn, which mediate local Ia inhibition to motoneurons (Hultborn et al 1971 a, Roberts 1972 b) also receive disynaptic Ia IPSPs. These are evoked from nerves to antagonist muscles of those supplied to the interneurons (Hultborn et al 1971 a, Hultborn 1972 and Schomburg unpublished). These Ia IPSPs are depressed by root volleys which indicates that Ia inhibitory interneurons to antagonistic motoneurons mutually inhibit each other.

5 To Ia primary afferents. Impulses in flexor nerves evoke primary afferent depolarization in Ia afferent terminals (Magni and Willis 1962) presumably through a polysynaptic pathway. The recurrent effects on the Ia interneurons in this preparation conditioning dorsal root potentials evoked by a short train in a flexor nerve (paper I). In no case were these dorsal root volleys affected by a single or a short train of ventral root volleys. This indicates that the Ia interneurons in the pathway from flexor afferent terminals do not receive recurrent inhibition from their collaterals.

B Selectivity in the recurrent control of Ia inhibitory interneurons

The question of a selective recurrent control of Ia inhibitory interneurons was recently discussed by Hultborn (1972) and the present study has brought some additional information. This will be summarized once more. The striking finding from the experiments (paper I) was that Ia IPSPs evoked in motoneurons were effectively depressed by volleys in the ventral root while IPSPs from Ib afferents or different types of Ia afferents were unaffected. Occasionally small polysynaptic IPSPs from high threshold muscle afferents and/or cutaneous afferents were depressed but the effects were always less pronounced than those of comparable size.

High threshold muscle afferents, cutaneous afferents, and high threshold joint afferents frequently evoke similar synaptic effects on motoneurons and are therefore often grouped together (FRA, R M Eccles and Lundberg 1959, Lundberg 1961). The FRA had been found to facilitate the activity of motoneurons.

inhibitory pathway to motoneurons (cf. Lundberg 1970) and to activate the recurrently inhibited Ia interneurons in the ventral horn (Hultborn *et al.* 1971a). Further the occasional depression of FRA IPSPs in the motoneurons was evoked from the same ventral roots as the depression of the Ia IPSPs in the cells. It was therefore suggested in paper I that the recurrently affected FRA IPSPs were partly mediated via the Ia inhibitory interneurons and thereby susceptible to recurrent depression.

This suggestion has received strong support from results reported by Fedina and Hultborn (1972 cf. also Hultborn 1972 a). They found in different types of preparations a strong positive correlation between the susceptibility of FRA IPSPs to recurrent depression and the ability of FRA volleys to facilitate the transmission in the Ia inhibitory pathway to motoneurons. Likewise the susceptibility of IPSPs evoked in motoneurons from different descending systems (cortico- rubro- and vestibulospinal tracts as well as a presumed reticulospinal pathway descending in the medial longitudinal fascicle) to recurrent depression correlates well with the ability of these systems to facilitate transmission in the Ia inhibitory pathway (Hultborn and Udo 1972 b c. Hultborn 1972 a). There is thus so far no examples of recurrent effects on IPSPs in motoneurons either from primary afferents or descending tracts which cannot be accounted for by an excitatory convergence from these systems onto the Ia inhibitory interneurons. All these findings would indicate that the recurrent effects on segmental reflex pathways to motoneurons are restricted to inhibition of the interneurons in the reciprocal Ia inhibitory pathway as suggested in paper I.

There seems to be one exception to this rule. Shortly after paper I was submitted for publication there appeared a paper by Ryall (1970) in which he gave evidence for mutual inhibition between Renshaw cells (cf. also Ryall and Piercey 1971. Ryall, Piercey and Polosa 1971. 1972). Previous scattered observations indicate that Renshaw cells can be fired also by other routes than the well known excitatory path from motor axon collaterals (Eccles *et al.* 1954, Frank and Fuortes 1956. Curtis, Phillis and Watkins 1961, Curtis and Ryall 1966). Accordingly it seems possible that they transmit inhibition from primary afferents which would thus be susceptible to recurrent depression. The fact that such a relay through Renshaw cells has not been needed to account for recurrent effects on different types of IPSPs may indicate either that the orthodromic excitation of Renshaw cells is very weak or that the mutual inhibition between them is weak (cf. Hultborn 1972 a). Since the present study started from the finding that the recurrent facilitation of motoneurons is a disinhibition, it might be appropriate to mention that Hultborn *et al.* (1971 c) found that this effect largely can be accounted for by the recurrent inhibition of the Ia inhibitory interneurons.

Turning to VSCT neurones the results with respect to recurrent effects on IPSPs from other afferent systems than the Ia are entirely in accordance with the findings from motoneurones (papers II and III). Thus Ib IPSPs were unaffected by ventral root volleys while ipsi- and contralateral FRA IPSPs were depressed in some cells. It is noteworthy that recurrent depression of FRA IPSPs were found only in VSCT cells with Ia IPSPs susceptible to recurrent depression and not in a large sample of VSCT neurones lacking such inhibition. In cells with recurrently affected FRA IPSPs it was possible to facilitate the Ia IPSPs from the FRA, just as was the case in motoneurones. Also some disynaptic descending IPSPs were recurrently depressed in cells with Ia IPSPs. In unanaesthetized preparations recurrent facilitatory potentials were evoked in VSCT neurones with Ia IPSPs but only from the ventral roots which depressed the Ia IPSPs in the cells. Since all these effects were found in cells lacking recurrent inhibition from motor axon collaterals the recurrent effects on interneuronal transmission can also in the case of VSCT neurones be explained by a selective action on the interneurones which mediate Ia inhibition.

The observation that dorsal root potentials evoked by Ia volleys were unaffected by ventral root stimulation (paper I; section A, 5) suggested that not all Ia excited interneurones were inhibited from Renshaw cells. Confirmatory evidence was obtained from recordings of monosynaptically Ia excited interneurones in the intermediate region which were found to lack recurrent inhibition (Hultborn *et al.* 1971 a). It is not known whether these cells are excitatory or inhibitory. However the existence of Ia inhibitory interneurones without recurrent inhibition is proved by the finding that Ia IPSPs in certain Ib VSCT neurones are unaffected by ventral root volleys (paper III; section A 2). This conclusion has been further supported by observations from DSCT neurones (section A, 3) and some group I interneurones in the intermediate region (section A, 4). Thus susceptibility to recurrent inhibition from motor axon collaterals is not a general property of Ia inhibitory interneurones.

C Is the Ia inhibition of VSCT neurones and α motoneurones mediated by the same interneurones?

The study of the recurrent control of the Ia inhibitory pathways to VSCT neurones was initiated in order to test the postulate that the Ia inhibition in these cells is evoked through collateral connections of the interneurones which relay Ia inhibition to motoneurones (Lundberg 1971). The most direct test would be to record from the interneurones and their

postulated target neurones in order to find if action potentials in the former give rise to unit IPSPs in both motoneurones and VSCT neurones. Even if simultaneous recordings from Ia inhibitory interneurones and their target motoneurones have been obtained (Jankowska and Roberts 1972 b) similar recordings from a triplet of neurones are hardly feasible especially when considering that Ia IPSPs are evoked only in some VSCT neurones. Any conclusions with respect to the Ia inhibitory relay to VSCT neurones therefore have to rest on indirect evidence as obtained in the present study.

Clearly the finding that the Ia IPSPs in many VSCT neurones are susceptible to recurrent depression from motor axon collaterals is in full accordance with the idea of a common interneuronal origin of the Ia IPSPs in VSCT neurones and motoneurones. The other possible explanation for the findings, namely two parallel Ia inhibitory pathways, one directed to the VSCT neurones and the other to motoneurones and both being recurrently controlled, is extremely unlikely (cf. paper II). Also from a functional point of view the idea of a common interneurone seems much more attractive. Even if the function of the recurrent control of the Ia inhibitory pathway to motoneurones is not fully understood this action presumably plays a role in the regulation of the depth of the reciprocal inhibition of motoneurones (Hultborn *et al.* 1972 b; Hultborn and Lundberg 1972; Hultborn 1971 a, b). The mere fact that the VSCT receives Ia inhibition which is regulated by the motor output in the same way as Ia inhibition to motoneurones strongly suggests that its information transfer is related to this inhibitory pathway. The most simple coupling would clearly be collateral connections from the interneurones in the Ia inhibitory pathway to motoneurones.

D Recurrent inhibition of VSCT neurones from motor axon collaterals

Since the findings with respect to the Ia inhibitory pathway to VSCT neurones were consistent with the idea that the VSCT conveys information about this pathway it was deemed important (cf. Discussion) to complete the picture by searching for recurrent inhibition of the VSCT neurones from motor axon collaterals. In paper IV was reported that IPSPs are indeed evoked in some VSCT neurones on antidromic stimulation of ventral roots. The IPSPs increased in amplitude with the α -volley had segmental latencies indicating a disynaptic linkage from the motor axons to the VSCT neurones and time courses similar to those of recurrent IPSPs in motoneurones and Ia inhibitory interneurones (cf. Eccles *et al.* 1954; Hultborn *et al.* 1971 a). It is thus likely that the pathway is similarly organized as the recurrent inhibitory path to the latter cells.

It is notable that all the recurrently inhibited VSCT neurones received monosynaptic excitation from Ia afferents and that in the Q excited cells the recurrent inhibition was evoked from the L5 and L6 ventral roots but not from the L7 and S1 ventral roots just as is the case for Q excited α -motoneurones and Ia inhibitory interneurones (paper I and Hultborn *et al* 1971a). Recurrent inhibition was found only in a very small fraction of the recorded Ia VSCT neurones but it should by no means be assumed that this connexion is aberrant (cf Discussion paper IV). Rather the small size of the pool of recurrently inhibited VSCT neurones is taken to indicate a high degree of selectivity in connections to individual VSCT neurones.

For the interpretation of the functional significance of the recurrent inhibition to VSCT neurones it is essential to learn whether it is mediated by the same interneurones which transmit recurrent inhibition to motoneurones and Ia inhibitory interneurones. It has earlier been mentioned that there is good reason to assume that the same Renshaw cells project to α -motoneurones and Ia inhibitory interneurones excited from Ia afferents in the same nerve. On the basis of the correspondence in the convergence onto the VSCT neurones and of similar functional considerations as in previous section it seems reasonable to postulate that the same Renshaw cells also project to VSCT neurones.

E Composition of the VSCT neuronal pool with respect to monosynaptic excitation from muscle afferents

The first studies on the VSCT led to the conclusion that the only primary afferents supplying monosynaptic excitation to VSCT neurones were the Golgi tendon organ (Ib) afferents (cf Oscarsson 1965b). This conclusion was based on mass discharge recordings from the tract (Oscarsson 1956, 1957), intraxonal recordings (Oscarsson 1957, Lundberg and Oscarsson 1962) and intracellular recordings from the cell bodies (Eccles *et al* 1961a). Of the VSCT neurones found in the latter two types of experiments a considerable proportion did not receive monosynaptic excitation from any of the dissected muscle or cutaneous nerves. The vast majority however received strong polysynaptic effects, predominantly inhibition, from the FRA. The cell bodies of the Ib VSCT neurones were found scattered in a region dorso-medially to the motor nuclei (Hubbard and Oscarsson 1962).

Later it has been found that a group of cells along the lateral border of the ventral horn, apparently identical with the spinal border cells of Cooper and Sherrington (1940) also belong to the VSCT (Burke *et al* 1971). A number of these cells are monosynaptically

excited from large muscle spindle (Ia) afferents while Ib excited cells are only occasionally encountered in this region (Lundberg and Weight 1971). Many of the spinal border cells are also without monosynaptic excitation from the commonly dissected hindlimb nerves (cf. Lundberg and Weight 1971 and paper II) and in experiments with stimulation of the dorsal columns it was proved that practically all the latter cells entirely lacked monosynaptic excitation from primary afferents (Lundberg and Weight 1971).

The sampling of different types of VSCT neurones in the studies of Eccles *et al* (1961a) and Lundberg and Weight (1971) probably was due to the use of different microelectrode approaches in the two studies. The insertion of the microelectrodes from the dorsal column (Eccles *et al*, (1961a) favoured the exploration of the region dorsomedially to the motor nuclei where the Ib cells are common, while the insertion of the microelectrodes laterally to the dorsal root entry zone (Burke *et al* 1971, Lundberg and Weight 1971) made the *spinal border cell region* more accessible.

In the present study (paper II) the microelectrodes were usually inserted from the dorsal column but with the knowledge of the more laterally located VSCT neurones they were angled to cover both the medial and lateral areas containing VSCT neurones. It is thus likely that the sampling of neurones in this case was more representative than in the earlier studies. Of more than 140 intracellularly recorded VSCT neurones about 25 per cent received mainly Ia excitation, 15 per cent mainly Ib excitation, 10 per cent group I excitation which could not be classified as evoked from either Ia or Ib afferents while about 45 per cent were without monosynaptic excitation from any of the dissected nerves. In a few additional per cent of cells there seemed to be convergence of monosynaptic excitation both from Ia and Ib afferents.

If anything the figure for cells without monosynaptic excitation from group I afferents is underestimated since these cells seem to be relatively more common in the L3 and L4 segments (Burke *et al* 1971) which were not so extensively explored in this study. The figure for Ib VSCT neurones would not be much underestimated since in the experiments especially devoted to record from Ib VSCT neurones (paper III) less than 40 per cent of the impaled cells received monosynaptic Ib excitation (Lindström and Schomburg unpublished). Provided no large group of VSCT neurones remains to be detected the results thus indicate that roughly a fourth of the VSCT neurones are monosynaptically excited from Ia afferents, another fourth monosynaptically excited from Ib afferents, while the remaining cells lack monosynaptic excitation from primary afferents.

These results should be correlated with anatomical findings of retrograde degeneration after spinal cord transections. The majority of cells showing retrograde changes after such operations are found along the lateral border of the ventral horn where the Ia VSCT neurones and those lacking group I excitation mainly are located, while only scattered cells showing chromatolysis are found in the more dorsal region where the Ib VSCT neurones are more common (Cooper and Sherrington 1940 Sprague 1953). With the reservation that not all cells with transected axons may show chromatolysis with the usual techniques, these findings seem to be in general agreement with the electrophysiological results.

When looking at the detailed pattern of effects to the VSCT it is apparent that each of the above groups consists of several subgroups. For instance, there are Ia VSCT cells which receive only Ia excitation or Ia excitation and inhibition from the same nerve, or reciprocally organized Ia excitation and inhibition or Ia excitation with recurrent inhibition from motor axon collaterals, or Ia excitation combined with Ib excitation (Lundberg and Weight 1971, papers II-IV). Each such subgroup may constitute a very small fraction of the total VSCT pool. The diversity would be even greater if effects from other afferent systems and descending tracts also were considered. This clearly indicates that each individual VSCT cell carries a very specific piece of information. It is also understandable that this highly differentiated input led Lundberg and Weight (1971) to conclude that one cell hardly resembles another.

GENERAL DISCUSSION

A Comments on the functional significance of the recurrent control of the Ia inhibitory pathway to motoneurons

The present results indicate that the recurrent control of reflex transmission from primary afferents to motoneurons is restricted to the interneurons in the inhibitory pathway from large muscle spindle (Ia) afferents to motoneurons. This conclusion has found strong support in subsequent studies in which also the recurrent control of descending effects was analyzed (cf Hultborn 1972 a). Since the recurrent inhibition of α -motoneurons is distributed primarily between motor nuclei coupled in Ia synergism (Eccles et al 1961 = Hultborn et al 1971 b) and since motoneurons and Ia inhibitory interneurons supplied with the same Ia excitation receive a similarly organized recurrent inhibition (Hultborn et al, 1971 b) it seems likely that the recurrent inhibition from motor axon collaterals is related to the γ -control of movements. This idea is supported by the recent finding that also some γ -motoneurons receive recurrent inhibition from motor axon collaterals (Ellaway 1968, Brown, Lawrence and Matthews 1968, Grillner 1968, Ellaway 1971, Noth 1971).

The idea that many movements depend on a coactivation of α - and γ motoneurons developed from the finding that several neuronal pathways evoke parallel effect in α - and γ motoneurons (Granit 1955, 1970, Grillner 1969, Matthews 1972). Such a coactivation is found in a number of natural movements: breathing (Eklund, Euler and Ruckowski 1964, Sears 1964), stepping (Severin, Orlovsky and Salk 1967), jaw movements (Taylor and Davey 1968) and voluntary finger movements in man (Hagbarth and Vallbo 1968, 1969, Vallbo 1971). In all these cases the balance between the α - and γ -activation is set so that the afferent discharge from the muscle spindles actually increases during muscle contraction. These findings indicate that the activation of motoneurons may depend on spatial facilitation between a more direct α -route of excitation and an indirect γ -route via the muscle spindles. In these movements the increase in spindle afferent discharges often begins after the onset of the α -activity and regular respiratory and walking cycles can occur also after deafferentation although the muscle strength is impaired. Accordingly these movements rather than being generated through a "follow-up length servo" operated by the γ -system (Morton 1953) can be considered to depend on α - γ -linkage and receive "servo assistance" from the γ system (cf Matthews 1972).

It is likely that reciprocal inhibition from Ia afferents plays a role in many types of movements. It has been pointed out that neuronal systems which supply parallel excitation to α - and γ -motoneurons also excite the interneurons in the Ia inhibitory pathway (Hongo *et al* 1969, Lundberg 1970). Thus the activity in the Ia inhibitory interneurons seems to depend on spatial facilitation between excitation through a more direct route, corresponding to the α -route to motoneurons and through the indirect γ -route via Ia afferents. These authors pointed out the advantage in certain situations of a parallel regulation of the excitation of α -motoneurons and of the interneurons relaying reciprocal inhibition to antagonist motoneurons. In order to stress the similarity in the regulation of the activity in motoneurons and Ia inhibitory interneurons they coined the term α - γ -linked reciprocal inhibition.

The need for reciprocal inhibition may vary in different types of movements and implicit in the idea of α - γ -linked reciprocal inhibition lies the possibility for control of reciprocal inhibition by other neuronal systems than Ia afferents. The interneurons interposed in the Ia inhibitory pathway may thus serve as an integrative station.

In order to understand the functional significance of Ia reciprocal inhibition it is necessary to consider the distribution of Ia actions. The "myotatic unit" in the terminology of Lloyd (1946 b) was originally believed to include only muscles acting as synergists and antagonists at the same joint. R. M. Eccles and Lundberg (1958 b) however found a wider distribution of Ia actions from some muscles and suggested that this extended Ia pattern has developed to assist locomotion (cf. also Engberg and Lundberg 1969, Lundberg 1969 b). In this connexion it is of interest that reciprocal Ia inhibition seems to operate only between muscles acting as flexors and extensors of different joints but not between abductors and adductors (R. M. Eccles and Lundberg 1958 b, Hongo *et al* 1969). The role of the reciprocal inhibition in the alternating activation of flexors and extensors in locomotion might be to eliminate stray excitatory effects during the passive phase of the step cycle and to cut off outlasting Ia excitation during the shifts between flexion and extension phases.

On the other hand, the wide distribution of excitatory and inhibitory Ia actions may rather be a disadvantage during more precise movements engaging only a single muscle or requiring cocontraction of muscles operating as antagonists at the same joint. Since the recurrent inhibition to motoneurons and Ia inhibitory interneurons "cover" this extended Ia pattern, Hultborn *et al* (1971 b) suggested that the role of the recurrent inhibition may be to limit the diverse Ia effects thereby subserving the γ -control of precise movements or postures, perhaps involving cocontraction of antagonist muscles. This idea presupposes that the Renshaw cells

can be controlled from other segmental and descending sources than the motor axon collaterals (for reference see paper IV) which provides a possibility to regulate the amount of recurrent inhibition produced by a given motor output

On the basis of the above consideration regarding the role of reciprocal Ia inhibition in stepping it is interesting that there is evidence for less effective transmission in the recurrent inhibitory pathway during stepping. In mesencephalic cats which perform locomotion on a treadmill during stimulation of the "locomotor region" (Shik, Orlovsky and Severin 1966) the recurrent inhibitory pathway to motoneurons is depressed during locomotion (Severin, Orlovsky and Shik 1968). In this connexion it also deserves to be mentioned that a similar depression can be evoked after DOPA (Bergmans, Burke and Lundberg 1969). This drug liberates transmitters from a descending noradrenergic pathway and thereby releases an interneuronal network with mutual inhibitory connections between interneuronal pools supplying excitation to extensors and flexors respectively (Jankowska, Jukes, Lund and Lundberg 1967). It has been suggested that this interneuronal network serves as the segmental generator for the alternating activation of flexors and extensors in locomotion. It has recently been found (Fu, Jankowska and Lundberg, unpublished observation) that FRA volleys which can activate either of these interneuronal pools also evoke excitatory action in the Ia inhibitory interneurons projecting to antagonist motoneurons (this pathway is not shown in Fig. 1). This finding provides another example of α - γ -linked reciprocal inhibition. After DOPA, FRA volleys may evoke reciprocal inhibition and it is worth noting that these reciprocal IPSPs are almost entirely removed by volleys in the appropriate ventral roots suggesting that the inhibition is mediated entirely through the Ia inhibitory interneurons. Thus associated with the activation of the spinal centres generating alternating activation of flexors and extensors there seems to be a regulation both of the pathway mediating reciprocal inhibition of motoneurons and of the recurrent inhibitory pathway controlling this reciprocal inhibitory pathway.

The parallel recurrent control to α -motoneurons and Ia inhibitory interneurons (presumably through the same Renshaw cells, Hultborn *et al.* 1971 b) should not be taken to indicate that the resulting effects necessarily are equivalent. It seems likely that the degree of reciprocal inhibition largely depends on regulation of the firing frequency of the Ia inhibitory interneurons while the regulation of the amount of motor activity largely depends on recruitment of new motor units (Severin, Shik and Orlovsky 1967, Grillner and Udo 1971). In case of motoneurons the recurrent inhibition evoked by the first mobilized cells will tend to increase the amount of excitation required to activate other motoneurons. The recurrent inhibition of the Ia inhibitory interneurons, on the other hand, may function by adjusting the average firing frequency

of the interneurons to a level appropriate for the intended movement. It is obvious that the suggested regulatory effect of the recurrent inhibition in relation to the reciprocal inhibition should not be seen as an all or nothing phenomenon. Even in cocontraction an appropriate balanced reciprocal inhibition might be desirable. For further aspects on the functional significance of the recurrent inhibition of Ia inhibitory interneurons see Hultborn and Lundberg (1972) and Hultborn (1972 a, b).

II Information transfer in the VSCT

The existence of two direct spinocerebellar pathways from the hind-limb region has been known since the end of the last century although there have been controversies with respect to the location of the cell bodies and the spinal course of the tracts (cf. Smith 1957, Jansen and Brodal 1958). It is now well established that the dorsal spinocerebellar tract (DSCT) contains neurones which are monosynaptically excited from afferents of muscle spindles, Golgi tendon organs, joint receptors and different types of cutaneous receptors (Oscarsson 1965 b, Lindström and Takata 1972); some of them receive convergence of excitation from different types of receptors while others seem to obtain a modality specific input. The excitation of the DSCT cells is often supplied from restricted receptive fields, for instance a single muscle or a part of a toe pad. Although a still poorly understood integration seems to occur at the DSCT cell level, this tract can be assumed to convey specific information from peripheral receptors to cerebellum.

The initial physiological studies on the ventral spinocerebellar tract (VSCT) by Oscarsson (1956, 1957) were concerned with neurones monosynaptically excited from Golgi tendon organ (Ib) afferents. These cells also received strong polysynaptic effects from the FRA and the FRA effects could be markedly influenced from several descending systems (Oscarsson 1965 b). Surprisingly and contrasting with the strong excitatory coupling to DSCT neurones the Ib VSCT neurones were very weakly driven by adequate activation of Golgi tendon organs of a single muscle (Oscarsson 1960). It was rather difficult to account for these findings and Oscarsson (1957, 1965 b) suggested in general terms that the VSCT neurones might forward information concerning movement or posture of a whole limb and that the descending control of the pathways from the FRA to VSCT neurones might allow selection of information from either tendon organ afferents or flexor reflex afferents.

With the finding that the spinal border cells of Cooper and Sherrington (1940) belong to the VSCT (Burke *et al.* 1971) it became clear that the VSCT consists of a nonhomogeneous population of neurones

some being monosynaptically excited from Ia afferents others from Ib afferents and some entirely lacking monosynaptic excitation from primary afferents (Lundberg and Weight 1971). The VBCT neurones also receive mono- and polysynaptic excitation and inhibition from several descending tracts (Oscarsson 1965 b; Baldissera and Bruggen cate 1969; Baldissera and Weight 1969). Lundberg (1971) noted a similarity in the convergence of excitation and inhibition from different types of primary afferents and descending tracts on segmental interneurons and on the VBCT neurones and forwarded the hypothesis that the VBCT relays information about the transmission in interneuronal reflex pathways to motoneurons. This hypothesis should be considered in the perspective of the extensive convergence from different segmental and descending systems on the interneurons in these reflex pathways as exemplified in Fig. 1 for the reciprocal Ia inhibitory pathway to motoneurons (cf. Hultborn 1972 a, b). It seems reasonable to assume that the descending control of motoneurons which is exerted mainly through such interneurons (Lundberg 1966, 1969 a; Hongo *et al.* 1972; Hultborn 1972 a, b) would require feedback information regarding the transmission in these pathways.

The idea that ascending pathways may relay information about interneuronal transmission to motoneurons is not new. The possibility has been discussed earlier in relation to effects from the FRA on ascending pathways (Lundberg 1959, 1964; Oscarsson 1967, 1968; Miller and Oscarsson 1970) among them the VBCT which receives strong FRA effects (Oscarsson 1965 b; Lundberg and Weight 1971). Lundberg's new hypothesis is, however, very attractive for the following reasons. Firstly, it offers a plausible explanation for the existence of two direct spinocerebellar pathways from the hindlimb region, the DSCT signalling information about the periphery and the VSCT about the activity in the spinal cord at the segmental level. Secondly, the hypothesis comprises one of the simplest and best known interneuronal reflex pathways in the spinal cord - the disynaptic Ia inhibitory pathway to motoneurons and is therefore accessible to experimental testing. Thirdly, the hypothesis gives a detailed account of possible mechanisms for the information transfer through the VSCT about interneuronal reflex pathways.

A central idea in the hypothesis is that VBCT neurones receive information about the activity in the interneuronal chains through collateral connexions, both from the last order interneurons in the pathways and from excitatory and inhibitory neurones impinging onto these interneurons. With respect to the Ia inhibitory pathway this idea has found strong support in the present study. It seems therefore appropriate to consider in some detail how the VSCT may convey information about interneuronal reflex pathways using the Ia inhibitory pathway as a model.

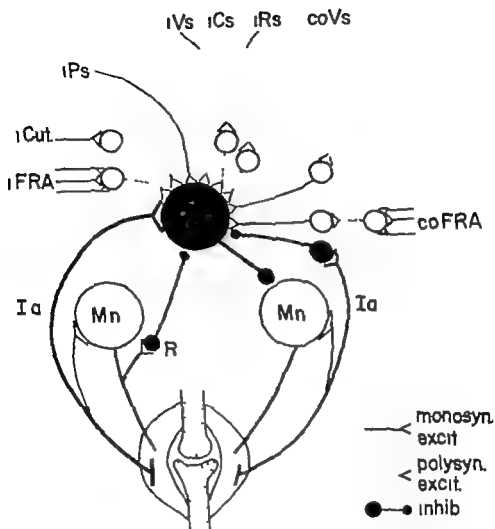


Fig 1 Schematic representation of some synaptic connections to the interneurons in the inhibitory pathway from large muscle spindle (Ia) afferents to α -motoneurons (cf Hultborn 1972 a, b) I, ipsilateral; co contralateral; Vs Cs, Rs Ps, vestibulo- cortico- rubro- and propriospinal tracts respectively; Ia, Ia afferents; Cut cutaneous afferents; FRA, flexor reflex afferents Mn motoneurons; R, Renshaw cells; ant, antagonist

For the descending regulation of a given Ia inhibitory pathway to motoneurons it should be of crucial importance to know the exact amount of inhibition which the interneurons in this pathway exert on their target motoneurons. This information could be obtained by collateral connections to VBCT neurones from the Ia inhibitory interneurons. However, since the output of these interneurons is in the form of inhibition, and the information has to be conveyed as spike activity, the inhibition has to be displayed against some background activity in the VBCT neurones. Such background activity could be generated by a pacemaker mechanism in the VBCT neurones or by some constant unspecific excitatory input to them but there are no indications of such arrangements for the VBCT. The problem seems to have been solved in the way that the VBCT neurones are supplied with the same Ia excitatory input as the Ia inhibitory interneurons again through collateral connections (Fig. 2 A). This is an input-output comparator cell for the Ia inhibitory pathway (Lundberg and Weight 1970, Lundberg 1971) and in reality this type of cell is represented by the VBCT neurones with convergence of monosynaptic excitation and disynaptic inhibition from Ia afferents in the same nerve (cf. paper II).

Such an input-output comparator cell might give an adequate information about the activity of the Ia inhibitory interneurons provided all excitatory and inhibitory neurones terminating on them also send collaterals to the VBCT cell. This seems, however, not to be the case, presumably because this information would be unsufficient for cerebellum. On the other hand, the information content in the firing of a single input-output comparator cell is considerably reduced if some of the inputs to the Ia inhibitory interneurons are missing to the VBCT cell. This is illustrated schematically in Fig. 2 B. Assume that only the Ia excitatory input is fed to the VBCT neurone together with the output from the interneurons. A certain relationship may then be found between the Ia excitation and the firing frequency of the input-output comparator cell as indicated by curve a. If the Ia inhibitory interneurons in addition are inhibited from some source, then the excitation of the VBCT neurone will be less effectively counteracted by the firing of the Ia inhibitory interneurons. The relation between the Ia input and the firing frequency of the VBCT cell will then change as shown by curve b. If the Ia inhibitory interneurons instead receive excitation from some source other than the Ia afferents they will fire more intensely for a given Ia input and give the relation depicted by curve c. Under such conditions it would be impossible to determine from the firing frequency of the input-output comparator cell if the Ia inhibitory interneurons receive a weak Ia excitation combined with inhibition (resulting in negligible Ia inhibition of the motoneurons) or a strong Ia excitation with additional excitatory impingements (giving profound Ia inhibition of the motoneurons, cf. dotted line in Fig. 2 B).

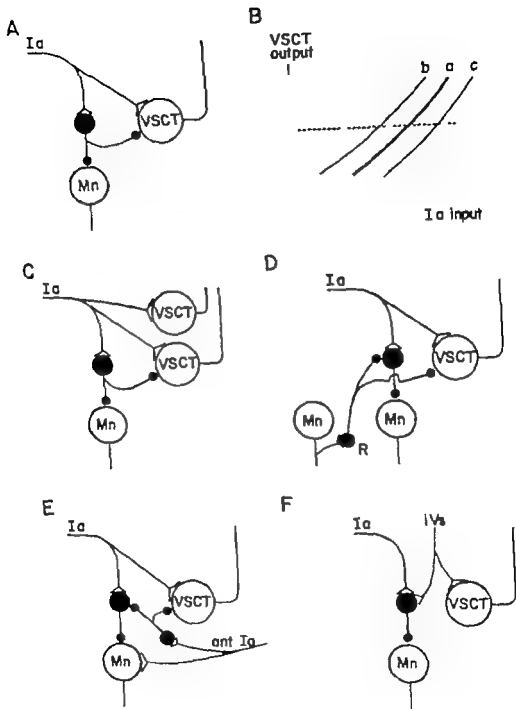


Fig 2 Schematic representation of different connexions to ventral spinocerebellar tract neurones. See text.

Such a difficulty might be overcome by having in parallel other input-output comparator cells which receive the same effects but in different quantities. A more interesting alternative, which is in keeping with experimental observations, is depicted in Fig. C. Parallel with the input-output comparator cell there is another VSCT neurone which receives only Ia excitation corresponding to that of the Ia inhibitory interneurons. By subtracting the firing of the input-output comparator cell from that of the other VSCT cell, cerebellum would obtain a measure of the inhibitory output from the Ia inhibitory interneurons. At the same time information about the Ia excitation supplied to these interneurons would be available.

Theoretically the Ia excitation and inhibition of the input-output comparator cells could be balanced so that the discharge frequency of these cells remained at a constant level independently of changes in the Ia excitation of the Ia interneurons (that is, curve a in Fig. 2 B could be horizontal). Any extra excitation of the Ia inhibitory interneurons from sources other than the Ia afferents would then be measured as a decrease in the firing frequency of the input-output comparator cells and inhibition of the interneurons would be measured as an increase in the frequency. However, since the Ia inhibition of the motoneurons varies with the Ia excitation of the interneurons, it would also in such cases be essential with an extra line to cerebellum for the Ia input, as shown in Fig. 2 C.

For a proper regulation of the Ia inhibitory pathway, cerebellum probably needs a detailed information also about the contribution of excitation and inhibition from other neuronal systems to the Ia inhibitory interneurons (cf. Fig. 1). Recurrent inhibition from motor axon collaterals and inhibition from other Ia inhibitory interneurons are two examples of such inputs to the Ia inhibitory interneurons. The present results indicate that information also about these effects may be available for cerebellum through the VSCT. Some VSCT neurones receive recurrent inhibition from motor axon collaterals and this effect is in all likelihood mediated through collaterals of Renshaw cells which project to the Ia inhibitory interneurons. Again the information is in the form of inhibition and also in this case the inhibition seems to be displayed against Ia excitation corresponding to that of the Ia inhibitory interneurons (Fig. 2 D; of paper IV). As in the case of the previously discussed input-output comparator cell, cerebellum could extract information about the amount of recurrent inhibition to the Ia inhibitory interneurons by comparing the output of this VSCT cell with that of a cell excited exclusively from Ia afferents. In a similar way information about the mutual Ia inhibition of the Ia inhibitory interneurons seems to be conveyed by VSCT neurones with reciprocity.

organized Ia excitation and inhibition (Fig 2 E; of paper II) The results with respect to other segmental inhibitory inputs to the Ia inhibitory interneurons are in principle compatible with the suggested scheme, but the organization of these inputs to the Ia inhibitory interneurons is not sufficiently well known to allow us to push the comparison any further (cf paper II)

Information about excitatory inputs to the Ia inhibitory interneurons is probably signalled in an analogous way There may be, however one important difference Since excitatory neurones through their collaterals will supply the VSCt cells with excitation, there would be no need to combine this input to the VSCt cells with Ia excitation This is exemplified in Fig 2 F with effects from the vestibulospinal tract which has been found to evoke monosynaptic excitation in some VSCt cells lacking effects from group I muscle afferents (Baldissera and Roberts: unpublished observation) However the vestibulospinal tract (unfortunately) has monosynaptic connections also with other segmental interneurons (Bruggencate, Burke Lundberg and Udo 1969) It is therefore impossible to tell whether VSCt cells like the one in Fig 2 F really are concerned with the reciprocal Ia inhibitory pathway since they lack the marking with Ia excitation The same difficulty would apply to other excitatory inputs to the Ia inhibitory interneurons On the other hand, VSCt neurones with Ia IPSPs susceptible to recurrent depression but without monosynaptic excitation from group I afferents (paper II) are likely to be related with some of the descending excitatory inputs to the Ia inhibitory interneurons

Certainly the above discussion gives a simplified description of the mechanisms for the transfer of information about the reciprocal Ia inhibitory pathway to motoneurons through the VSCt For instance, cells can be found which in different combinations receive convergent effects corresponding to several of the inputs to the Ia inhibitory interneurons (cf Fig 2 paper IV) However the pattern of convergence on VSCt neurones found in the present study shows that the VSCt has the capability to convey a detailed information about the different inputs to and the output from the interneurons in the reciprocal Ia inhibitory pathway to motoneurons thereby giving a complete account of the inhibitory control of motoneurons through this pathway The results also indicate that this information is relayed fractionated through a set of VSCt neurones each one carrying a part of the total information

Lundberg (1971) discussed at some length how the VSCt may convey information about other inhibitory reflex pathways to motoneurons for instance from Ib afferents or the FRA More direct experimental evidence for an information transfer through the VSCt about these other pathways is still scanty mainly because the organization of these pathways is not known in sufficient detail to allow appropriate testing However

Baldissera and Roberts (unpublished observation) have obtained results indicating that some VSCT neurones are concerned with the last order inhibitory interneurones in the inhibitory pathway to flexor motoneurones from the contralateral FRA which also receive monosynaptic excitation from the vestibulospinal tract. Paper III described some observations indicating that VSCT neurones may receive convergence of monosynaptic excitation and disynaptic inhibition from Ib afferents in the same nerve, giving Ib input-output comparator cells. On the assumption that Ib excited VSCT neurones may be concerned with Ib pathways to motoneurones and from the observations that these VSCT neurones frequently receive Ia inhibition (Eccles et al 1981 a paper III) it was also suggested that the interneurones in the Ib pathways may be under inhibitory control from Ia inhibitory interneurones. If this prediction should prove to be correct it would provide further evidence in support for the discussed VSCT hypothesis. It is noteworthy that Ia and Ib afferents, which are the only primary afferents known with certainty to have disynaptic connections with motoneurones, likewise are the only afferents found to connect monosynaptically with the VSCT.

Although Lundberg (1971) mainly discussed the VSCT function in relation to inhibitory reflex pathways it should not be excluded that some VSCT neurones may convey information about excitatory interneuronal pathways for instance from Ib afferents or from the FRA. In such cases an input-output comparator arrangement would not be required since the excitatory interneurones in these pathways can activate directly the VSCT neurones through collateral connections, as discussed above for excitatory neurones impinging on the Ia inhibitory interneurones. As in that case it might be very difficult to obtain experimental evidence for this possible VSCT function. Information about excitatory pathways to motoneurones would be obtained also if the convergence on some VSCT neurones was related to the convergence on motoneurones instead of interneurones (Lundberg 1971). It should be mentioned that since recurrent inhibition and reciprocally organized Ia excitation and inhibition are evoked both in Ia inhibitory interneurones and motoneurones the VSCT cells with the convergence shown in Fig. 2 D and E may as well signal information about excitatory and inhibitory actions on motoneurones.

In papers II and III the claim of Arshavsky, Berkintblit, Gelfand, Orlovsky and Fukeon (1972a) that the rhythmic modulation of the discharges in VSCT neurones in walking cats remains after deafferentation was considered as a support for the hypothesis that the VSCT is concerned mainly with interneuronal activity at the segmental level. Unfortunately the criteria used by these authors for identification of the VSCT neurones, as described in the full papers (Arshavsky et al. 1972b c) leave some doubt whether the investigated cells really belong to the VSCT. It seems therefore necessary to repeat these very interesting experiments.

with properly identified units. It would certainly also be interesting to know whether the present hypothesis about the function of the VSCT is applicable on the forelimb equivalent - the rostral spinocerebellar pathway (Oscarsson and Uddenberg 1964, 1965; Oscarsson 1965 a)

At present very little can be said about the cerebellar processing of the information received through the VSCT. The finding that each VSCT axon can be antidromically activated at low threshold from a relatively large area of the cerebellar cortex (compared to the DSCT - cf. Lundberg and Oscarsson 1960, 1962; Burke *et al.* 1971) - suggesting an extensive preterminal branching of the VSCT mossy fibres - may be of relevance in relation to the suggested organization of the VSCT with sets of neurones relaying fractionated information about a given reflex pathway. The bilateral termination in the cerebellar cortex of some VSCT neurones (Lundberg and Oscarsson 1962; Burke *et al.* 1971) may be correlated with the bilateral termination in the spinal cord of some segmental interneurones. Needless to say the information about central activities at the segmental level received by cerebellum through the VSCT should not be seen in isolation from information supplied through other channels for instance from the periphery through the DSCT. However the intricate organization of the cerebellar cortex with a vast divergence and convergence in the mossy fibre-Purkinje cell loop (Palkovits, Magyar and Szentágothai 1972) implies that the cerebellum is capable of "decoding" a complicated information submitted through a multiplicity of individual input neurones.

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SUMMARY AND CONCLUSIONS

- 1 The effects of impulses in recurrent motor axon collaterals on transmission in inhibitory reflex pathways from different types of primary afferents to α -motoneurons and ventral spinocerebellar tract neurones have been investigated in the cat by conditioning IPSPs evoked in these neurones
- 2 The transmission in the disynaptic inhibitory pathway from large muscle spindle (Ia) afferents to α -motoneurons of antagonist muscles is inhibited by antidromic impulses in motor axons. The effect is due to postsynaptic inhibition of the interneurons interposed in this pathway and is mediated through recurrent motor axon collaterals and Renshaw cells. The results indicate that besides Renshaw cells themselves these interneurons are the only segmental interneurons influenced from motor axon collaterals through Renshaw cells
- 3 The transmission in the disynaptic Ia inhibitory pathways to some (but not all) ventral spinocerebellar tract neurones is also inhibited at the interneuronal level by antidromic impulses in motor axon collaterals. It is concluded that the Ia inhibition of these cells and of α -motoneurons is mediated by the same interneurons
- 4 Disynaptic recurrent inhibition from motor axon collaterals is evoked in some ventral spinocerebellar tract neurones monosynaptically excited from Ia afferents. The effect is presumably mediated through collateral connexions from the same Renshaw cells which inhibit α -motoneurons and Ia inhibitory interneurons
- 5 The results with respect to the ventral spinocerebellar tract strongly indicate that this tract is capable of signalling information about the transmission in segmental reflex pathways to α -motoneurons. Possible mechanisms for this information transfer are discussed, using the Ia inhibitory pathway as a model

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Reflex Organization and Contraction Properties of Facial Muscles

An experimental study in the cat

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Reflex organization and contraction properties of facial muscles

This review is based on investigations described in the following papers which are being referred to by their Roman numerals

- I Mechanisms involved in the cat's blink reflex. *Acta physiol scand* 1970 80 149—159 (together with A. Mårtensson)
- II Facilitation and inhibition of facial reflexes in the cat induced by peripheral stimulation. *Acta physiol scand* 1972 85 126—135
- III Analysis of facial reflex facilitation and inhibition by microelectrode recording from the brain stem. *Acta physiol scand* 197 85 183—192
- IV Contraction properties of cat facial muscles. *Acta physiol scand* In press.
- V Histochemical fiber composition of some facial muscles in the cat in relation to their contraction properties. *Acta physiol scand* In press. (together with L. Edström)

In the review of the individual papers some recent reports by other investigators which were not available when the original papers were published will also be considered.

Before summarizing the results of the individual papers a more detailed background for the experiments not made room for in the original papers will be presented in the Introduction.

Introduction

As compared with the extensive work on reflex control and physiology of extremity muscles very little interest has been taken in the reflex organization and contraction properties of facial muscles. Our knowledge of facial reflexes is based almost exclusively upon clinical observations, whereas there are few experimental studies on animals. Considering that the human facial reflexes have been incorporated into the routine examination of the nervous system it should be desirable to know more about the peripheral origin and the control of facial reflexes. There is also very scanty information on the contraction properties of the facial muscles proper and nothing seems to be known about their relationship to the histochemical fiber composition. These problems can most easily be tackled in animal experiments and the cat was chosen for this purpose in the experiments to be summarized below. Comparisons of results obtained from experiments on cats and man seem to be justified also from a phylogenetic point of view according to the work of Huber and Hugtson (1926). They showed that the facial muscles of mammals are derived from two primitive muscle layers: the platysma and the sphincter colli profundus. From these primitive muscles there has been a differentiation and specialization along different lines within various groups of mammals. However, the muscles studied in the present thesis: the orbicularis oculi, the orbicularis oris, the quadratus labii superioris and the caninus muscles are all derived from the sphincter colli profundus and may be identified both in cat and man.

Reflex contractions of human facial muscles were described in the late nineteenth century. Reflex blinking in response to a tap over the supraorbital region of the face was noted by Overend (1896) and Eschench (1898) showed that reflex contractions of the lip musculature could be produced by a tap on the upper lip. These reflexes were subsequently studied by a number of clinicians and described under various names according to the presumed origin of the reflex afferents or by the name of the discoverer. Overend interpreted the blink in response to a tap as a true skin reflex, but Wartenberg (1945) refuted the concept that it should be a defense reaction and proposed that it might be set up by stretch activation of intramuscular receptors. He called it a myotatic reflex for which he suggested the name: the orbicularis oculi reflex. Thus far the ideas advanced regarding the origin of facial reflexes in man had been based on purely clinical observations but in the early fifties Kugelberg and coworkers (Kugelberg 1952, Ekblom, Jernelius and Kugelberg 1952)

approached the problem by means of electromyographic analysis. They were able to show that tap-elicited reflex contractions of the human orbicularis oculi as well as of perioral muscles are actually composed of two different reflex discharges, one of short latency and one of longer latency. They arrived at the conclusion that the early reflex components were monosynaptic "myotatic" reflexes and the late components nociceptive reflex responses. This seemed to reconcile the divergent opinions prevailing as to the nature of the reflexes. However Kugelberg himself drew attention to the fact that muscle spindles had not been found in facial muscles of man or other mammals. This uncertainty still prevailing as to the peripheral origin of the facial reflexes initiated the experiments presented in paper I.

As early as in Overend's work it had been suggested that the afferent link of the blink reflex was in the primary division of the trigeminal nerve. By electrical stimulation of trigeminal afferents Kugelberg (1952) could show that this applies to both reflex components. That afferents in the trigeminal nerve are involved in facial reflexes is now well established. However some observations indicate that afferents in the facial nerve may also play a part in the elicitation of the early (Rushworth 1962) as well as the late component (Gandiglio and Fra 1967) of the human blink reflex. From histological studies of the cat's facial nerve we know that a small percentage of its thin myelinated afferent nerve fibers take their origin in the mimic muscles (Broesch 1944). Their possible role in the reflex control of the facial muscles is also considered in paper I.

The animal experiments performed on the facial reflexes are chiefly those of Tokunaga and coworkers (Tokunaga, Oka, Murao, Yokio, Okumura, Hirata, Miya-shita and Yoshitatsu 1958) who took up investigations on the central pathways of the double trigemino-facial reflexes. They showed that the early reflex component was absent following lesions of the ipsilateral trigeminal main sensory nucleus and that its threshold was lowered when the contralateral half of the midbrain was transected. The long latency reflex component was absent following the latter procedure and also following lesions to the contralateral ventral thalamic nucleus. Ipsilateral trigeminal tractotomy or destruction of the trigeminal main sensory nucleus raised the threshold for but did not abolish the late reflex component. The conclusion was drawn that the respective reflex pathways pass through the regions where lesions cause a decrement of the respective reflex. The possibility was not considered that systems having a facilitatory or inhibitory influence on the reflex chains may have been damaged. However since the work of Tokunaga and coworkers was presented a number of studies of the sensory impulse flow in trigeminal afferents have been published. It has been shown that there is a control of the trigeminal afferent inflow from suprabulbar as well as from peripheral sources (for review see e.g. Darian-Smith 1966). The importance of this sensory gate for the control of facial reflex activity has not been investigated. The interaction between various peripheral sources in the

control of facial muscle reflex activity is therefore considered in paper II and further analyzed in paper III

But little attention has been given to the contraction properties of the facial muscles only single observations of contraction times in man have been reported. However it may be of interest to recollect that the Swedish histologist Häggqvist (1940) undertook studies of the efferent innervation of the facial muscles from which he made implications concerning their physiology. He found that the facial nerve in rhesus monkeys and cats is made up of small-diameter nerve fibers. He also identified motor nerve endings of the type later shown to be typical of slow tonic non-twitch muscle fibers, but this finding could not be confirmed in a recent work by Hess (1962). The small motoneurons in the spinal cord seemed specifically concerned with the maintenance of skeletal muscle tone and consequently the facial muscles should have "a pronounced tonic mode of working" (Häggqvist 1940). Even though it has later been shown that the small motoneurons of the spinal cord have the lowest threshold for recruitment in the stretch reflex (Henneman, Somjen and Carpenter 1965) it is not a corollary that the same relationship between nerve morphology and muscle function applies to the facial muscles. An answer to this question can only be given by studying the contraction properties of the facial muscles, and results from such experiments are given in paper IV.

Ever since Ranvier in the late nineteenth century observed that there are functional differences between "red" and "white" muscles the possibilities of correlating structure, biochemistry and muscle function have considerably increased. In particular with the advent of histochemical techniques it has become possible to correlate characteristics in metabolism of a muscle fiber to certain contraction properties. The availability of such methods is utilized in paper V in an attempt to form a more coherent picture of the facial muscle reflex system.

Results and Discussions

Paper I. Mechanisms involved in the cat's blink reflex

This paper deals with four specific questions: 1) does tap stimulation of the cat's face give rise to reflexes similar to those evoked in man? 2) how is the double facial muscle reflex discharge produced? 3) are muscle spindles engaged in the reflex control of facial muscles or do other types of end-organs serve this purpose? 4) do facial nerve afferents play a part in the reflex control of facial muscles?

The first approach to these problems was obviously to create an experimental situation similar to that used by Kugelberg in his human experiments. Thus, tap stimulations were applied to different facial areas and the EMG led off from the orbicularis oculi and the perioral muscles. Particular attention was given to the reflex discharges of the orbicularis oculi since the most constant responses were recorded from this muscle, and it was found that also in the cat a double reflex discharge results on tap stimulation of the facial skin. The early response had a latency range of 9–12 ms and the late component a range of 15–25 ms. As in the human experiments the reflex responses evoked on tapping at sites remote from the eye were weaker than those evoked from sites close to the eye. There were some observations that did not seem to fit in with the concept that the early reflex component was generated by activation of muscle spindles. Its latency variations were greater than should be expected from a monosynaptic reflex, it was very frequency-sensitive, it had a fairly long duration and above all it could be evoked by tapping a skin flap dissected free from the underlying muscle but not by tapping the muscle proper. The multisynaptic origin of the early reflex component was supported by estimates of its central relay time which gave values of 4 ms. The mechanisms responsible for the late component were also studied and the conclusion was drawn that there is a separate reflex chain but that peripheral mechanisms may be operative in its potentiation. Thus, an off volley may be set up by reactivation of skin receptors when the stimulus probe is withdrawn from the skin and if the stimulus is brief enough this off volley may via a short latency reflex transmission, occur at the moment when the long latency reflex could be expected. Furthermore, skin receptors may be activated by the early reflex contraction and thus reactivate the short latency reflex chain, resulting in a double reflex discharge.

Recently Shahani (1970) and Shahani and Young (1972) reinvestigated the human orbicularis oculi reflex and arrived at the conclusion that also in man the two components of the reflex are elicited by activation of mechanoreceptors in the facial skin.

Although it proved impossible to evoke monosynaptic facial reflexes, attempts were made to record afferent impulses from muscle spindles or other intramuscular receptors. These attempts were unsuccessful even though succinylcholine was used as a potent stimulator of intrafusal muscle fibers. There was thus further reason for the opinion that the facial muscles lack a conventional proprioceptive reflex control system. However it was found that even a very small muscle contraction may give rise to a marked reflex contraction by activation of skin receptors discharging through trigeminal nerve afferents. It was suggested that such afferent discharges may be of some importance for the proprioceptive control of phasic facial muscle contractions. This concept has gained support from recent experiments on human subjects with and without local anesthetic block of trigeminal afferents (Leanderson and Persson 1972)

Weak electrical stimulation of the peripheral facial nerve branches elicited reflexes in various facial muscles. These reflexes disappeared on section of the appropriate trigeminal nerve branches and could not be recorded from the facial nerve in curarized cats. Thus, no evidence for the presence of large-diameter afferents in the facial nerve was found. However when a stronger electrical stimulation was applied to the ventral branch of the facial nerve a reflex discharge with a latency of 25 ms could be recorded in the nerve to the orbicularis oculi even in the curarized cat. The threshold for these reflexes was around six times that of maximal activation of the facial motor nerve fibers, and it was therefore concluded that these reflexes must have been set up by activation of the thin myelinated facial nerve afferents (cf. above). The reflexes were not identical with the tap-elicited facial reflexes since no afferent discharge could be recorded in the facial nerve in response to tap stimulation.

Paper II. Facilitation and inhibition of facial reflexes in the cat induced by peripheral stimulation

In this paper the effects of conditioning stimuli on the reflexes studied in paper I are described and analyzed. (A further analysis of the conditioning effects is given in paper III.) Reflexes set up in the orbicularis oculi and caninus muscles by tap stimulation of the face were subjected to conditioning stimuli of various types. When the conditioning stimulus was a facial tap reflex facilitation resulted when the conditioning test interval was less than 20 ms, whereas with intervals between 40 and 80 ms reflexes of lower amplitude were obtained. Reflex inhibition was observed even with conditioning test intervals up to 450 ms. A reflex depression was also noted when the conditioning tap was given to contralateral areas. It was therefore unlikely that the inhibitory effects were due to a skin deformation persisting from the conditioning facial tap. This could also be ruled out by the fact that facilitation

as well as inhibition of facial reflexes could be obtained also by an electrical conditioning stimulation of trigeminal nerve afferents. It was concluded that central nervous mechanisms were involved in the interaction phenomena. It was also found that electrical stimulation of afferents in various other peripheral nerves could condition the tap-elicited reflexes. Thus electrical stimulation of lingual and hypoglossal nerve afferents resulted in periods of facilitation and inhibition whereas inhibition invariably resulted on conditioning by stimulating afferents in the ipsilateral radial or saphenous nerves.

The time course of the inhibitory effects suggested presynaptic inhibition. To test whether pre- or postsynaptic inhibition was involved, drugs were injected for selective blocking of one or the other type of inhibition, picrotoxin being used to block the presynaptic and strychnine to block the postsynaptic type. These experiments showed a clear difference between the inhibitory effect elicited by conditioning stimuli from distant sites such as radial nerve afferents and inhibition induced by facial conditioning taps, in that the latter but not the former was diminished by strychnine. The effects of picrotoxin were equivocal. The conclusion was drawn that postsynaptic inhibition is involved when the conditioning stimulus is a tap to the facial skin. The reflex depression evoked from distant sites was considered to be due to presynaptic inhibition because of its longer duration and its resistance to strychnine. Presynaptic inhibition was a likely explanation also for the late part of the long-lasting depression induced by conditioning facial taps.

In man, habituation of the blink reflex results on repeated stimulation. A small series of experiments was made to find out whether this is the case also in the anesthetized cat and if possible to find an explanation for the phenomenon. The tap-elicited blink reflex was found to be considerably depressed even at a frequency of 4/s which agrees well with the findings in man. Since habituation occurred also in decerebrate cats, cortical mechanisms, which have been widely accepted as the cause of blink reflex habituation in man, could be ruled out. Instead it was suggested that presynaptic inhibition at the brain stem level should be involved since the interval between the taps at the lowest stimulus frequency producing habituation was approximately similar to the longest interval at which the test reflexes were depressed in the conditioning test experiments.

Paper III. Analysis of facial reflex facilitation and inhibition by microelectrode recording from the brain stem

In this paper a further step in the analysis of the reflex control of facial muscles is taken by recording events in the facial motor nucleus with extracellular microelectrodes. The compound antidromic response resulting from stimulation of various peripheral facial nerve branches is described. The effect on these potentials of

conditioning antidromic facial nerve volleys was then studied with the particular view in mind to elucidate whether the motor impulse flow from facial motoneurons like that from the spinal motoneurons is controlled by an inhibitory (or facilitatory) feed back mechanism from motor axon collaterals (Renshaw inhibition). It was found that the excitability of the motoneuron pool was regularly depressed for 30 ms and sometimes even for up to 100 ms following antidromic invasion. In contrast to the Renshaw type of inhibition at the spinal level this decreased excitability was unaffected by injections of strychnine. It was therefore concluded that it was due to post-spike hyperpolarization of motoneurons rather than to feed-back inhibition.

In a short communication Kitai and coworkers have reported a similar interaction of antidromic volleys in the facial nucleus of the cat. They also made intracellular recordings from some motoneurons and found a duration of after-spike hyperpolarization of up to around 30–40 ms. To account for the longer periods of depressed excitability in the motor nucleus they postulated some inhibitory mechanism. Strychnine was not used in their analysis (Kitai Akaike Bando Tanaka, Tsukahara and Yu 1971).

In paper III the effects on the motoneuron pool of trigeminal synaptic activation are also analyzed in order to see to what extent the facilitatory and inhibitory phenomena described in paper II occur in the facial motoneurons. The field potential evoked in the facial nucleus by electrical stimulation of peripheral trigeminal nerve branches is described. The amplitude of the antidromically elicited potential in the facial nucleus was taken as an index of the current state of excitability of the motoneurons. The results showed that there is an increased motoneuron excitability 3–35 ms following a facial tap stimulation and a period of lowered motoneuron responsiveness from 35 to 65 ms following the facial conditioning tap. The initial period of antidromic potential facilitation was interpreted as being due to excitatory postsynaptic potentials set up by the afferent volley in facial motoneurons and the period of lowered excitability as coincident with a period when facial motoneurons are under postsynaptic inhibitory influence. Further support was thus given to the idea put forward in paper II that postsynaptic inhibition is involved in the control of facial muscle reflex excitability.

The trigeminal synaptic input to the facial nucleus has now been recorded intracellularly from facial motoneurons by Tanaka, Yu and Kitai (1971). In their study excitatory postsynaptic potentials on electrical stimulation of peripheral trigeminal nerve branches were found to occur in facial motoneurons with a minimum latency of 2–3 ms, which should correspond to an input by di- or polysynaptic pathways. Their paper and the results reported here thus support the findings presented in paper I showing that trigemino-facial reflexes are not monosynaptic.

In paper III discharges from reticular neurons in response to stimulation of facial and hypoglossal nerve afferents are also described. The latencies of the responses of

these neurons and their discharge properties were similar to those of neurons responsible for the N_2 component of the trigeminal field potential and their location in the brain stem similar. The N_2 component is related to depolarization of trigeminal afferent terminals in the brain stem. It was therefore suggested that facial and hypoglossal nerve afferents may exert a depolarizing action on afferent trigeminal brain stem terminals by these reticular neurons and thus produce presynaptic inhibition of facial reflexes.

Paper IV: Contraction properties of cat facial muscles

Facial muscles have been studied from various aspects but very little attention has been paid to the physiological characteristics of the effector organs *i.e.* to the contraction properties of the muscles. Values of contraction times occasionally recorded in, *e.g.* the human frontalis muscle (McComas and Thomas 1968) indicate that they are fast. As mentioned in the introduction it has been inferred from histological studies that the facial muscles have a tonic mode of functioning which is usually characteristic of the slow "red" striated muscles. Such a mode of action is however inconsistent with the morphology of the facial muscles in which no muscle spindles could be identified in the electrophysiological experiments described in paper I or in the histological studies presented in paper V. In fact, it is difficult to set up a tonic discharge in facial muscles even in a decerebrate cat (unpublished observation). The experiments described in paper IV were undertaken to study the contraction properties of the facial muscles, the main interest being focused on the orbicularis oculi and oris. These muscles were chosen because minimal surgery is required and the risk of injury to the superficial and delicate muscle fibers thus small. A comparison of the contraction properties of the two muscles is of interest also because they differ functionally the orbicularis oculi having, in contrast to the orbicularis oris, a very low threshold for reflex activation (cf. paper I).

Maximal isometric contractions in response to motor nerve stimulation were recorded from the orbicularis oculi and oris as well as from the depressor conchae. The average contraction times were found to be 8.5 ms, 33 ms and 24 ms respectively. The three muscles may thus all be classified as fast twitch muscles. When electrical stimuli of increasing strength, from threshold to supramaximal values, were applied to the motor nerves of the orbicularis oculi and oris, no appreciable differences could be observed in the time course of the resulting twitches. The motor units in the respective muscles should thus have uniform twitch contraction characteristics.

The responses of the orbicularis oculi and oris to repetitive nerve stimulation were studied as well. Summation of individual twitches was found to start at a frequency around 20/s in the former muscle but in the latter at a frequency as low as around

conditioning antidromic facial nerve volleys was then studied with the particular view in mind to elucidate whether the motor impulse flow from facial motoneurons like that from the spinal motoneurons is controlled by an inhibitory (or facilitatory) feed-back mechanism from motor axon collaterals (Renshaw inhibition). It was found that the excitability of the motoneuron pool was regularly depressed for 30 ms and sometimes even for up to 100 ms following antidromic invasion. In contrast to the Renshaw type of inhibition at the spinal level this decreased excitability was unaffected by injections of strychnine. It was therefore concluded that it was due to post-spike hyperpolarization of motoneurons rather than to feed-back inhibition.

In a short communication Kitai and coworkers have reported a similar interaction of antidromic volleys in the facial nucleus of the cat. They also made intracellular recordings from some motoneurons and found a duration of after-spike hyperpolarization of up to around 30–40 ms. To account for the longer periods of depressed excitability in the motor nucleus they postulated some inhibitory mechanism. Strychnine was not used in their analysis (Kitai Akaike Bando Tanaka, Tsukahara and Yu 1971).

In paper III the effects on the motoneuron pool of trigeminal synaptic activation are also analyzed in order to see to what extent the facilitatory and inhibitory phenomena described in paper II occur in the facial motoneurons. The field potential evoked in the facial nucleus by electrical stimulation of peripheral trigeminal nerve branches is described. The amplitude of the antidromically elicited potential in the facial nucleus was taken as an index of the current state of excitability of the motoneurons. The results showed that there is an increased motoneuron excitability 5–35 ms following a facial tap stimulation and a period of lowered motoneuron responsiveness from 35 to 65 ms following the facial conditioning tap. The initial period of antidromic potential facilitation was interpreted as being due to excitatory postsynaptic potentials set up by the afferent volley in facial motoneurons and the period of lowered excitability as coincident with a period when facial motoneurons are under postsynaptic inhibitory influence. Further support was thus given to the idea put forward in paper II that postsynaptic inhibition is involved in the control of facial muscle reflex excitability.

The trigeminal synaptic input to the facial nucleus has now been recorded intracellularly from facial motoneurons by Tanaka, Yu and Kitai (1971). In their study excitatory postsynaptic potentials on electrical stimulation of peripheral trigeminal nerve branches were found to occur in facial motoneurons with a minimum latency of 2–3 ms, which should correspond to an input by di- or polysynaptic pathways. Their paper and the results reported here thus support the findings presented in paper I showing that trigemino-facial reflexes are not monosynaptic.

In paper III discharges from reticular neurons in response to stimulation of facial and hypoglossal nerve afferents are also described. The latencies of the responses of

these neurons and their discharge properties were similar to those of neurons responsible for the N_2 component of the trigeminal field potential and their location in the brain stem similar. The N_2 component is related to depolarization of trigeminal afferent terminals in the brain stem. It was therefore suggested that facial and hypoglossal nerve afferents may exert a depolarizing action on afferent trigeminal brain stem terminals by these reticular neurons and thus produce presynaptic inhibition of facial reflexes.

Paper IV Contraction properties of cat facial muscles

Facial muscles have been studied from various aspects but very little attention has been paid to the physiological characteristics of the effector organs, i.e. to the contraction properties of the muscles. Values of contraction times occasionally recorded in, e.g. the human frontalis muscle (McComas and Thomas 1968) indicate that they are fast. As mentioned in the introduction it has been inferred from histological studies that the facial muscles have a tonic mode of functioning which is usually characteristic of the slow "red" striated muscles. Such a mode of action is however inconsistent with the morphology of the facial muscles in which no muscle spindles could be identified in the electrophysiological experiments described in paper I or in the histological studies presented in paper V. In fact, it is difficult to set up a tonic discharge in facial muscles even in a decerebrate cat (unpublished observation). The experiments described in paper IV were undertaken to study the contracting properties of the facial muscles, the main interest being focused on the orbicularis oculi and oris. These muscles were chosen because minimal surgery is required and the risk of injury to the superficial and delicate muscle fibers thus small. A comparison of the contraction properties of the two muscles is of interest also because they differ functionally the orbicularis oculi having, in contrast to the orbicularis oris, a very low threshold for reflex activation (cf. paper I).

Maximal isometric contractions in response to motor nerve stimulation were recorded from the orbicularis oculi and oris as well as from the depressor conchae. The average contraction times were found to be 8.5 ms, 33 ms and 24 ms respectively. The three muscles may thus all be classified as fast twitch muscles. When electrical stimuli of increasing strength, from threshold to supramaximal values, were applied to the motor nerves of the orbicularis oculi and oris, no appreciable differences could be observed in the time course of the resulting twitches. The motor units in the respective muscles should thus have uniform twitch contraction characteristics.

The responses of the orbicularis oculi and oris to repetitive nerve stimulation were studied as well. Summation of individual twitches was found to start at a frequency around 20/ in the former muscle but in the latter at a frequency as low as approx.

10/s. No further increase in tension was observed in the orbicularis oculi at frequencies above 100/s and in the orbicularis oris above 60/s there is thus a wider working range for the orbicularis oculi. The relation between the maximal tetanic contractions and the twitches was 7.0 and 4.5 respectively.

In an attempt to find an explanation for the large differences in contraction times between the two muscles, the duration of the maximal intensity of the active state in a single twitch was estimated using three different, well-known techniques (cf. paper IV). Nerve stimulation was used. Since this does however not give a synchronous activation of the muscle which is needed for exact measurements, it is possible that small differences between the muscles were not disclosed. The range of the values found was 3.5 to 5 ms for both muscles, differences that might account for the dissimilarities in contraction times were thus not found in these experiments. According to Hill's (1949) generally accepted theory the fall of the active state curve from its maximum passes through the peak of the twitch curve, and since the tetanus/twitch ratios of the two muscles was known, it was suggested that differences in the rate of fall of the active state may be one factor accounting for the different contraction times. The experiments did however not yield any information about the "intrinsic speed of shortening" of the muscles or about the behavior of their series-elastic elements during contraction, which are the other two factors determining the contraction time of a muscle. However a difference in the "intrinsic speed of shortening" should be accompanied by differences in the activity of myofibrillar ATPase (cf. Close 1972) and no such difference was observed in the histochemical studies presented in paper V.

The first component in the human blink reflex does not to any great extent contribute to the downward movement of the eyelid according to some investigators (Shahani and Young 1972). On the other hand, there are findings indicating that one and the same motor unit may discharge both in the short and the long latency blink reflex component (Shahani 1970). In order to seek a clue to these somewhat discrepant findings, the EMG was recorded simultaneously with the actual contraction in a tap-elicited blink reflex. It was then found that a contraction produced by the short latency reflex component alone attains its maximum in around 15 ms and that it may be 2—3 times larger than a maximum twitch. These findings indicate that fast motor units discharging repetitively are recruited in the early reflex response. The contraction elicited by a double reflex discharge reached a peak in around 30 ms indicating that the prolongation of the reflex contraction must have been produced by motor units about as fast as the units contracting in the early response since the latency of the second reflex response is 15—25 ms (see paper I). A difference in function between the two reflex components was thus not apparent from the investigations presented in this paper and in paper I. It was however observed that a double reflex is most readily evoked on tapping in the close vicinity of the eye.

(see paper I) and this may imply that the function of the long latency reflex is to prolong and strengthen the blink reflex when a noxious object threatens the eye

Paper V Histochemical fiber composition of some facial muscles in the cat in relation to their contraction properties

An increasing number of papers are being devoted to studies of enzyme activity and substrate content in skeletal muscle by histochemical techniques since it has proved possible to relate the results of such investigations to functional properties of the muscles. In particular the activity of myofibrillar ATPase seems to be higher in fast muscles and the activity of oxidative enzymes higher in muscles with a well developed capacity for prolonged work.

In the experiments described in paper V muscle biopsies were taken from the orbicularis oculi, the depressor conchae and the orbicularis oris muscles and immediately frozen in liquid nitrogen. The material was then stained by various methods to show the activity of myofibrillar ATPase, the two oxidative enzymes succinate dehydrogenase and NADH₂ tetrazolium reductase and the glycolytic enzyme phosphorylase as well as the content of glycogen and fat. The three investigated muscles all contained muscle fibers with a high (type II) and such with a low (type I) activity of myofibrillar ATPase. The former type made up 90 % of the fiber population in the orbicularis oculi 85 % of the fibers in the orbicularis oris and 70 % of those in the depressor conchae. Considering the large difference in contraction times between the orbicularis oculi and oris it was surprising to find an almost equal number of fibers with a high activity of myofibrillar ATPase in the two muscles. This could be interpreted in one of two ways either there is no direct correlation between the intrinsic speed of shortening and the activity of myofibrillar ATPase or the differences in contraction time are due to other factors than to dissimilarities in the intrinsic speed of shortening. One such factor may be a difference in the decay of the active state, as pointed out in paper IV and this may in turn be due to differences in the rate of uptake of calcium into the sarcoplasmic reticulum, which may be independent of the myofibrillar ATPase activity.

The facial muscle fibers with a low activity of myofibrillar ATPase (type I) seem to be equipped for an aerobic type of metabolism. Thus the activity of the oxidative enzymes was high or intermediate, as was also the fat content, whereas the glycogen content and phosphorylase activity was low. This conforms to the histochemical characteristics of type I fibers in extremity muscles.

The fibers with a high activity of myofibrillar ATPase (type II) have a high activity of phosphorylase and a high glycogen content, which is typical of such fibers also in extremity muscles. There was always a correspondence between the

fat content and the activity of the oxidative enzymes thus, if the fat content in a fiber was low intermediate or high the enzyme activity was also low intermediate or high respectively. The predominant type of fiber in this group had an intermediate content of fat and an intermediate activity of oxidative enzymes in the orbicularis oris, but in the other two muscles a low oxidative enzyme activity and a low fat content. Thus among the facial muscle fibers with a high activity of myofibrillar ATPase there are those equipped for aerobic and anaerobic metabolism and those designed only for anaerobic metabolism. The latter fibers are white in appearance and the former red.

Since the orbicularis oris is composed mainly of fibers with an intermediate staining reaction for oxidative enzymes it should be expected to perform better than the orbicularis oculi in prolonged work. This was tested by stimulating the motor nerves to the two muscles at 5—20/s for various lengths of time and recording the resulting twitch tension decline. In comparison with a prestimulus twitch elicited at 1/s there was on the average a 30 % loss of twitch tension in the orbicularis oculi after stimulation of the muscle at 5/s for 10 min whereas only 13 % of the twitch tension had been lost in the orbicularis oris. Muscles subjected to such periods of prolonged stimulation were examined histochemically after the experiment and the activity of succinic dehydrogenase was found to be similar to that in an unstimulated muscle, but the glycogen had been depleted from many fibers in the orbicularis oculi. The glycogen depletion was primarily confined to muscle fibers with a low activity of succinic dehydrogenase. Thus, these experiments confirm the previous finding that there is a relationship between glycogen depletion and muscle fatigue and they validate the assumption that the orbicularis oris performs better than the orbicularis oculi during prolonged work.

Papers IV and V show that the facial muscles do not form a functionally homogeneous group but that there is a differentiation of their contraction properties which is accompanied by differences in enzyme activity and substrate content.

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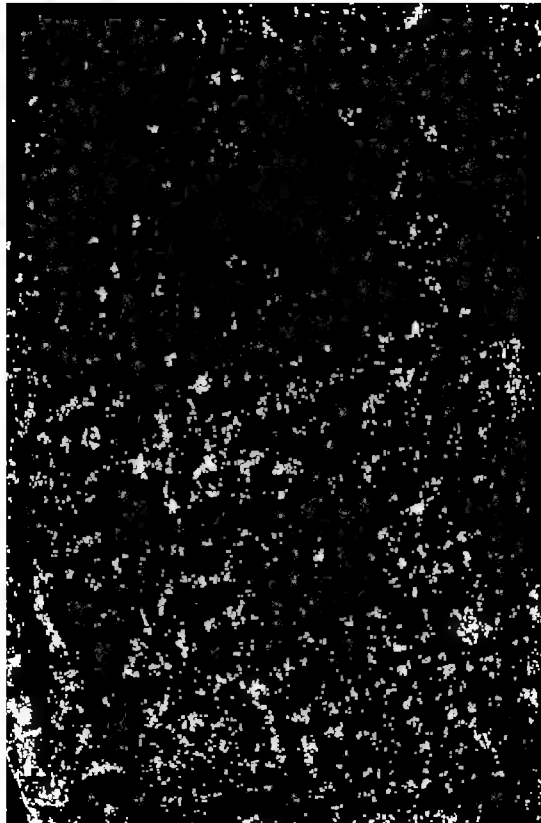
DEVELOPMENT OF SOMATOSENSORY
CORTICAL FUNCTIONS

AN ELECTROPHYSIOLOGICAL STUDY IN PRENATAL SHEEP

BY

HANS E. PERSSON

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FROM THE DEPARTMENT OF PHYSIOLOGY

KAROLINSKA INSTITUTET

STOCKHOLM 80, SWEDEN

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INTRODUCTION

The development of functions of sensory systems was originally analysed from the appearance and changes of the reflexes which could be elicited by afferent stimulations (for reviews, see e.g. Windle 1941 Carmichael 1951 1954 Gottlieb 1971). Several basic developmental concepts were postulated such as the functional precocity of somesthesia, particularly the trigeminal afferent inflow compared to audition and vision.

The introduction and use of electrophysiological techniques, notably the evoked potential method made it possible to obtain further information about the functional development of the sensory systems. The investigations on somatosensory evoked cortical responses in newborn dogs, cats, rabbits and rats (Scherrer and Oeconomos 1954 Grossman 1955 Marty 1962 Delhay-Bouchaud 1964 Thairu 1971 Verley and Rokyta 1972) give conclusive evidence that the afferent connections from the skin receptors to the cortical projection areas are already present at birth in these animals. Recent studies on the visual system during postnatal development have provided data on the involvement of different subcortical and cortical structures in the generation of various components of the evoked cortical response (Rose 1968 a,b 1971 Rose and Lindsley 1968). Comparable investigations have not been made on the maturation of somatosensory cortical responses. However the relative functional importance of the afferent pathways directed towards the somesthetic cortex of postnatal animals can be deduced from findings on electrocortical activities in primary and nonprimary projection areas evoked by electrical stimulation of ventrobasal and intralaminar thalamic nuclei and reticular structures (Purpura 1961 a,b, 1962, Scheibel 1962, Scheibel and Scheibel 1964 1971 Verley Siou and Garma 1966, Verley 1967 a, Verley and Siou 1967). In addition, responses have been described in subcortical relay stations evoked by peripheral tactile stimulation (Verley 1967 b).

The importance of the number and speed of the neuronal signals for the functional differences between the neonatal and the mature afferent systems has recently been discussed (Scherrer Verley and Garma 1968, 1970). Interest has also been paid to the ability of the immature sensory cortex to code information concerning different qualities of the peripheral stimuli. Thus,

there are data available on the postnatal development of receptive field characteristics in the visual cortex (Hubel and Wiesel 1963 Wiesel and Hubel 1963 Barlow and Pettigrew 1971) and of somatotopic organization in the somesthetic cortex (Rubel 1971) The results indicate that the functional organization of the cells within the somesthetic cortex, which serve the processing of afferent information is fairly well organized at birth in postnatal cats. No investigation has been directed towards the problem of the building up of this functional organization during the early stages of ontogeny

Several studies on mammals employing natural and electrical stimulation of different peripheral receptors have demonstrated qualitative and quantitative changes in the electrocortical responses as a function of age (Hunt and Goldring 1951 Scherrer and Oeconomos 1954 Rose, Adrian and Santibanez 1957 Ellingson and Wilcott 1960 Marty 1962 Marty and Thomas 1963 Delhaye-Bouchaud 1964 Pujol Granier and Marty 1966 Molliver 1967 Fox 1968 Mysliveček 1968 a, Pujol and Marty 1968 Rose 1968 a,b 1971 Rose and Lindsay 1968 Meyerson and Persson 1969 Pujol 1971 Sedláček 1971 Thairu 1971 Briquel and Verley 1972 Persson and Stenberg 1972 Rose, Gruenau and Spencer 1972 Verley and Rokyta 1972) In newborn dogs, cats, rabbits and rats stimulation in the periphery of the somatosensory system generates activity in the cortex as represented by a long-latency surface negative response (Scherrer and Oeconomos 1954 Grossman 1955 Marty 1962 Delhaye Bouchaud 1964 Thairu 1971) On the basis of studies on effects of pharmacological agents on gross surface responses in the somatosensory cortex of neonatal kittens it has been suggested that both excitatory and inhibitory synaptic processes are involved at different levels of the cortex (Purpura 1961 c Purpura Shofer Housplan and Noback 1964) The relative importance of inhibitory synaptic mechanisms in the newborn feline cortex has been repeatedly stressed (Purpura, Shofer and Scarff 1963 Purpura 1969 1971) Correlative morphological studies show that the cortical cytoarchitectonics at this neonatal stage are already relatively differentiated with well separated layers containing neurons in different phases of maturation (e.g Cajal 1960 Noback and Purpura 1961 Marty 1962) In addition, electron microscopic investigations on the cortex of perinatal dog cat and mouse have demonstrated a characteristic abundance of axodendritic synapses in the superficial cortical layers (Voeller Pappas and Purpura 1963 Meier Bleipohl and Clee 1968 Molliver and van der Loos 1970 Adinolfi 1971 1972) The prominent superficial neuropile with well-developed axodendritic synapses has been considered to account for the predominating surface negative evoked and spontaneous electrocortical activities in the neonate (Marty

Chevreau and Scherrer 1961 Marty 1962 Purpura *et al* 1964) During the late postnatal period the somesthetic evoked cortical potential undergoes a change into its adult positive-negative form. The involvement of basilar dendrites and axosomatic synapses (Marty *et al.* 1961 Marty 1962 Purpura *et al.* 1964) and a deep neuropile with specific corticopetal fibers as well as dendrites and axonal network of interneurons (Scheibel 1962 Scheibel and Scheibel 1964 1971) has been regarded to be of importance for bringing about this change.

The data described indicate that the somatosensory afferent pathways and their cortical projections have reached a comparatively high degree of structural and functional maturation in the neonate of the aforementioned animals. Consequently it is of interest to analyse the initial phases of functional development in the somatosensory system during the early prenatal period.

The classical investigations by Barcroft and Barron (1939 a,b 1941 1942) on externalized sheep fetuses kept in umbilical contact with the ewe, indicates that this preparation would be appropriate for the electrophysiological exploration of the immature central nervous system. This technique was adopted by Bernhard, Kaiser and Kolmodin (1959) and several investigations from this laboratory have shown that this preparation is suitable for the electrophysiological analysis of the development of spinal and cortical functions from very early stages of ontogeny (Bergström, Bernhard and Änggård 1960 Änggård, Bergström and Bernhard 1961 Bernhard, Kaiser and Kolmodin 1962 Änggård and Ottoson 1963 Edelberg Kolmodin and Meyerson 1965 1967 Kolmodin and Meyerson 1966 Bernhard Kolmodin and Meyerson 1967 Molliver 1967 Bernhard and Meyerson 1968 1973 Meyerson 1968 a,b Meyerson and Persson 1969 1973 Persson 1971 1973 Bernhard Meyerson and Persson 1972 Persson and Stenberg 1972) Since the sheep is relatively mature at birth from a neurophysiological and behavioral point of view (*c/* Ruckebusch 1971) the entire cycle of development of various nervous functions can be conveniently studied under the same physiological and technical conditions before a transition takes place from intruterine to extrauterine life. In addition, basic data on the neuroanatomical development of the isocortex in fetal lambs has been presented by Åström (1967)

It has been demonstrated that the somesthetic evoked cortical response in fetal sheep undergoes a series of changes as a function of age (Molliver 1967) The characteristic surface positive wave form of the somesthetic evoked potential in immature fetal sheep (see also Meyerson and Persson 1969 Persson 1971 1973) compared to the predominately negative response of neonatal animals (*e.g.* Scherrer and Oeconomos 1954 Marty 1962 Thairu 1971) indicates that a different mode of cortical activation exists in the

sensory cortex during the early prenatal stages of development. The knowledge about the cortical generative mechanisms to this immature cortical activity and its structural correlates is sparse.

The present investigation on the development of somatosensory cortical functions during early prenatal ontogeny deals mainly with the following aspects

- 1 The functional development in the somatosensory cortex and its morphological correlates.
- 2 The development of the specific and nonspecific somatosensory systems.
- 3 The mode of operation in the immature somatosensory system.

These aspects have been studied by means of analysis of gross and unit cortical responses evoked by tactile trigeminal stimulation. The changing characteristics of these responses have been correlated to simultaneous alterations of the cortical morphology

Preliminary reports derived from this study have been presented (Meyerson and Persson 1969 Persson 1971 1973)

METHODS

I. Electrophysiology

1 Material

The results are based on 67 experiments performed on sheep fetuses of different Swedish breeds (Gotland breed and Swedish landrace). The gestational age of the experimental animals was known in most cases and in the remainder estimated from weight-age diagrams (Meyerson 1968 b). Fetal ages ranged between 42 days and full term, i.e. about 145 days.

2 Animal preparation

The details of the animal preparation have previously been reported (Meyerson 1968 b) and only the general outline of preparation and the control of animal condition will be presented.

On the morning of the experimental day the pregnant ewe was fastened to an operation table and anesthetized with a short acting barbiturate Thiogonal® (Merck Darmstadt) or Pentothalsodium® (Abbott) 25–30 mg/kg slowly injected into a cannulated foreleg vein. The general anesthesia was maintained throughout the surgical procedure with repeated injections (5–10 mg/kg). The need for additional anesthesia was judged from the corneal reflex.

The animal was tracheotomized and artificially ventilated with a respirator (Harvard respirator Model 613). In most of the experiments the CO₂-content of the expired air was measured (Beckman gas analyzer Model LB-1) through a small tube inserted into the tracheal cannula and continuously monitored on a Grass-polygraph. The respirator was adjusted to maintain a level of 4–4.5% CO₂ in the end-tidal volume. Respiratory minute volumes were 50 to 100 l depending on the weight of the ewes (*cf* Cross, Dawes and Mott 1959).

A catheter was inserted into the proximal end of one of the ligated common carotid arteries for continuous recording of the arterial blood pressure and the pulse rate. The systolic and diastolic pressure was generally about 120 and 90 mm Hg respectively and the pulse rate varied between 60 and 120 per min (*cf* Spector 1956). Care was taken to maintain the temperature of the ewe at its normal value of 39 °C (*cf* Clawson 1928).

In order to avoid bronchospasm and pulmonary atelectasis an injection of adrenaline (0.2—0.4 mg s.c.) was given the animal was hoisted to an upright position a ventriculotomy was performed and the stomach contents evacuated.

The ewe was immobilized with Flaxedil® (Gallamine triethiodide May and Baker) 2—3 mg/kg i.v. and decerebrated by a section placed at the level of the posterior thalamus. This operation completed the surgical procedure of the ewe and the general anaesthesia was now discontinued. Throughout the experiment small doses of Flaxedil® were administered. In the dog, it has been shown that gallamine is present in fetal blood after the injection of large doses (20—50 mg/kg) into the maternal uterine artery (Pitinger and Morris 1955) However with the small doses used in the present study there was no sign of any placental transfer of the drug to the fetus.

After about two hours of recovery the fetus was carefully delivered through a caesarian section. The fetus was placed on a plastic cushion and embedded in thin cotton sheets soaked in warm mineral oil. Great care was taken not to damage the cotyledons or to stretch the umbilical cord. The body temperature of the fetus was continuously monitored with a rectal and a skin thermistor. The normal temperature (39°C) of the fetus was maintained with the aid of a heating lamp above the fetus and warm water running in the plastic cushion. Two silver pins were placed in the forelegs and connected to a Grass-polygraph for continuous recording of the fetal EKG. The pulse rate of the younger fetuses (60—80 days) was 160—200/min but was lower in the fetuses near term (*cf* Barcroft and Barron 1945).

The head of the fetus was then secured to a specially designed headholder. Under a dissecting microscope (Zeiss Epitechnoscope) a small or wide, unilateral or bilateral craniotomy was made according to the purpose of the experiment. The dura was reflected. The exposed cortical surface was covered with thin polyethylene sheets and frequently flushed with warm mineral oil.

The majority of the fetuses exhibited spontaneous mobility (*cf* Barcroft and Barron 1939 a,b 1942) and were therefore immobilized with a small dose of Flaxedil® 1—3 mg/kg intraperitoneally. It has been demonstrated in the adult cat, that gallamine has excitatory effects on cuneate neurons (Galindo Krnjević and Schwartz 1968) on thalamic relay nuclei neurons (Andersen and Curtis 1964) and on cortical excitability (Halpern and Black 1967). These effects were attained with much larger doses (> 6 mg/kg) than those used in the present investigation. No significant effect on the experimental results from the use of gallamine could be observed.

3 Stimulation

As in the adult animal (Nougier 1963) ipsilateral upper lip stimulation in sheep fetuses elicited cortical responses with the most constant form, shortest latency and largest amplitude and was therefore used in the present study.

Tactile stimulation was performed with a spherical probe, which had a diameter of 0.5 mm, connected to an electromagnetical transducer driven by a stimulator (Grass). One or two tactile stimulators were used each mounted on specially constructed coordinate systems allowing movements in all directions. The stimulation profile was a square wave of variable duration and amplitude. In most experiments the duration of the stimulus was 10 msec. The amplitude of the stimulation was chosen so as to provide reproducible cortical responses.

Sheep fetuses have glabrous nose skin until a gestational age of about 90 days when the first hairs appear on the nose. Not until shortly before full term do the fetuses have well-developed fur. Due to this fact the tactile stimulation was on bare skin in most experiments but in some older fetuses also on skin hairs.

4 Recording

Recordings were made of evoked gross potentials from the surface and the depth of the cortex as well as of evoked unitary activity from single neurons.

The surface exploring, reference and ground electrodes were all matched calomel half-cells connected to the preparation by agar saline bridges in polyethylene tubes with a recording area of 0.5 mm². The reference electrode was placed on saline moistened cotton in direct contact with the posterior part of the skull. The ground electrode was placed on a strip of saline moistened cotton wrapped around the neck.

The electrodes were connected to a Grass P 6 amplifier and a Tektronix 502 CRO D.c. recording with an upper cut-off frequency of 0.5 or 2 kHz was used in most experiments. The drift of this system was generally less than 50 μ V per hour. In some experiments a.c. recordings were used with a time-constant of 100 msec. A Grass kymograph camera provided the photographic recording.

Evoked field potentials and extracellular single unit activity were recorded with glass microelectrodes filled with sodium chloride (5.5 N) or potassium chloride (2.8 N). It is obvious that leakage of KCl from the micropipettes could affect the single unit activity. However no change could be detected in the firing pattern of spontaneously active neurons, which were extracellularly recorded with KCl microelectrodes during 15–20 minutes. Micro-

(or both) of the surface response. Furthermore on the basis of the depth potential profiles estimates were made of the corresponding vertical current gradients to achieve additional data on the location of sources and sinks'

II. Histology

After completion of the experiment, specimens were taken for histology from the somerthetic cortex of 11 fetuses (62 to 127 days of gestation) The tissue samples were fixed in formalin (10% in Runger's solution) and stained with cresyl violet. In 8 brains the shrinkage caused by the preparation was estimated (see pp 40)

RESULTS

I. Development of somatosensory evoked surface responses

Cortical responses to tactile stimulation were obtained in all 67 fetuses examined. During development the cortical evoked response to ipsilateral upper lip stimulation underwent a series of characteristic changes in cortical distribution, configuration, latency amplitude and ability to follow repetitive stimulation. Basic data on the maturation of the somesthetic evoked surface cortical response in fetal sheep have been given in an initial study from this laboratory by Moliver (1967). The present investigation constitutes a confirmation and extension of this work mainly in order to provide a firm basis for the understanding of the depth potential data.

1 Cortical distribution

The distribution on the cortex of the evoked response to tactile stimulation of the ipsilateral superior lip (ISL) was studied in fetuses of various ages. It was observed that a change of the stimulus locus from one point to another on the nose induced a shift in the cortical area from which the maximal responses could be obtained. This indication of a somatotopic organization was found even in the youngest age group. Thus, in a 69-day-old fetus the response could be recorded from an anterior and lateral area which included approximately the whole anterior third of the limencephalic brain (Fig 1 A). It is interesting to note this relatively large response area and the similarity between this ISL-representation and that of the adult sheep (Adrian 1943; Hatton and Rubel 1967).

The finding in all fetuses that the responses obtained from the periphery of the cortical receiving area had the same onset latency as those in the center of the field indicates that the fringe responses do not represent electrocortical activity due to a time-consuming tangential intracortical activation.

In Fig 1 B is shown the response distribution in a 76-day-old fetus and it can be seen that the cortical response was recorded from a large area of the anterior pole of the brain. A shallow impression on the lateral aspect of the brain denoted the appearance of a *suprasulcus* (cf. Meyerson 1968 b). No responses were obtained from recordings medial to this sulcus, i.e. from the anlage of *g. sup. asylvius* (association cortex) nor from the prospective *g. frontalis superior* (motor cortex).

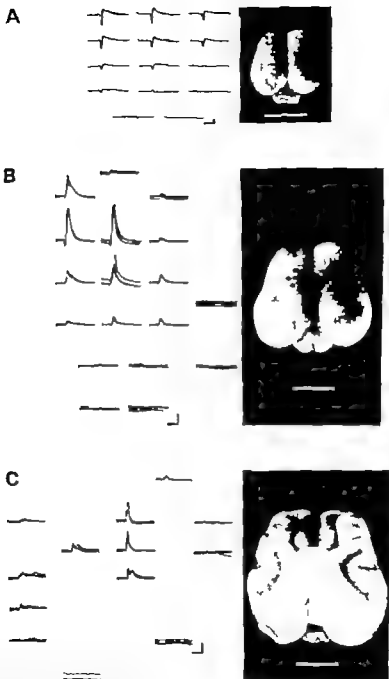


Fig. 1. Distribution of cortical responses evoked by tactile stimulation of the ipsilateral superior lip. A: 69-day fetus; B: 76-day fetus; C: 91-day fetus. The records of the cortical responses correspond to the relative positions of dots on the brain. Horizontal bars below the brains represent 1 cm. Calibration: 200 μ V, 100 ms. In this and subsequent figures negativity is upwards.

After the age of about 85 days, it is possible to trace the adult pattern of sulci and convolutions in fetal brains (Meyerson 1968 b). The somesthetic evoked response in a 91-day-old fetus could be recorded from *g. frontalis medialis* and *g. ectosylvius anterior* (Fig. 1C). These two areas presumably correspond to SI and SII respectively in the adult animal (Woolsey and Fairman 1946). No responses were obtained from *g. suprasylvius* to tactile stimulation of the nose.

2 Configuration

The change in the configuration of the somatosensory evoked cortical response is a prominent developmental feature. Fig. 2 shows superimposed cortical potentials ($n = 3-16$) evoked by tactile stimulation of the ipsilateral upper lip in fetuses of various ages. A well-defined response was obtained even in

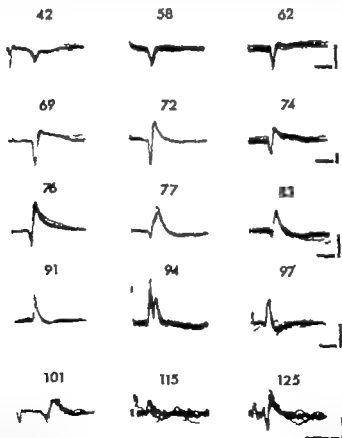


Fig. 2. Development of the configuration of evoked cortical responses in fetuses of various ages. The numbers above each record represent fetal ages in days. Calibration: 200 μ V, 100 ms.

the youngest fetus examined (42 days 12 g Fig 2, 4?) In this as in the other fetuses younger than 68 days (Fig 2 58 and 62) the response had a surface positive form. This unique observation that the somesthetic response of sheep at its appearance in early ontogeny displays a surface positivity was first described by Molliver (1967) At a fetal age of about 68—70 days a small negativity appeared in the response after the positive deflection (Fig 2 69) During the following developmental stage between about 70 and 80 days (Fig 2, 72 74 76 77 and 83) this negativity increased in amplitude and successively became the predominating component. During the same period the initial positivity showed a decreasing amplitude. In most fetuses of 90—100 days, the evoked cortical potential was characterized by a single surface negativity (Fig 2 91) sometimes followed by a small positive wave (Fig 2 97) However in a few fetuses responses consisting of a double negative peak were encountered (Fig 2 94) Predominating negative evoked responses to peripheral stimulation have also been recorded from the somesthetic cortex of cats (Scherrer and Oeconomos 1954 Marty 1962) rabbits (Marty 1962 Delhay-Bouchaud 1964 Verley and Rokyta 1972) and rats (Thairu 1971) during the immediate postnatal period of development. Around 100 days of fetal life in the sheep a small positive component, preceding the negativity reappeared in the response (Fig 2 101) This positivity increased in relative amplitude with age (Fig 2, 115 and 125) and at about 125 days the somatosensory cortical response displayed a positive-negative configuration similar to that of the adult animal (compare the α -response of Nougier 1963)

3 Latency

The development of the onset latency of the evoked cortical potential and the peak latencies of its main components is shown in Fig 3 A—D The onset latency of the initial positive component amounted to 115 msec in the youngest fetus (42 days Fig 3 A) This onset latency value should be compared to 0—25 msec obtained in the fetuses near term. The corresponding values of the peak latencies of the positive component were 150 msec and 30 msec respectively (Fig 3 B) The peak latency of the negative component in 69-day-old fetuses amounted to about 160 msec (Fig 3 C) The same value in fetuses older than 120 days were 40—50 msec. The latencies of the different components of the cortical response were, thus about 4—5 times longer in the younger fetuses as compared with the corresponding values in the fetuses near term (Fig 3 D) For comparison, the onset and peak latency of the positivity and negativity in adult sheep on electrical stimulation of the ipsilateral superior lip have been reported to amount to 5 12 and 17 msec respectively (Nougier 1963)

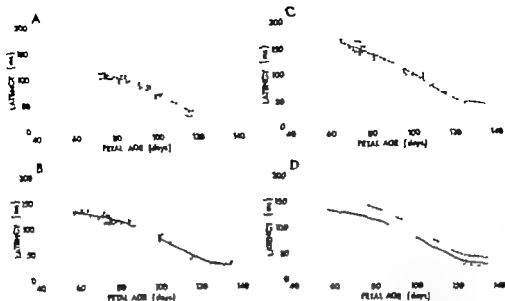


Fig. 3. Changes with age of onset and peak latencies of the evoked cortical response. A, onset latency (dots, dashed-and-dotted curve) B, peak latency of the positivity (open squares, solid curve); C, peak latency of the negativity (triangles, dashed curve) D the developmental curves of onset and peak latencies shown together (symbols as in A—C). Curves are fourth order polynomial approximations to the experimental data.

The graph in Fig. 3 A (see also Fig. 3 D) illustrating the development of the onset latencies of the somesthetic response, shows a plateau at about 110 msec between 40 and 80 days of age. There is a marked decrease of latency between the 80th and the 120th day of gestation after which the latency levels off. The peak latencies showed a continuous decrease to about the 120th day and then a levelling off (Fig. 3 B C see also Fig. 3 D).

4 Amplitude

When the intensity of the tactile stimulation was increased stepwise above the threshold, the peak amplitude of the cortical response increased in each step and finally reached a level of saturation. The range in response amplitude from the threshold to the saturation level had a tendency to be larger in younger than in older fetuses. The data plotted in Fig. 4 illustrating the development of the peak-to-peak amplitude of the evoked potential were derived from responses obtained with supramaximal stimulation. The maximal responses in the fetuses younger than about 63 days were of low amplitudes (150–600 μ V). Between 65 and 76 days, cortical responses could be obtained with peak-to-peak amplitudes of up to 1500 μ V. However there was

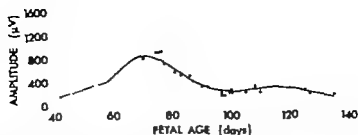


Fig. 4 Development of the peak-to-peak amplitude of the evoked cortical response. Curve is eighth order polynomial approximation to the experimental data.

a large variability in the amplitude of the responses recorded from fetuses of that age and low-amplitude evoked potentials were also observed. During the following developmental period the cortical responses showed a progressive diminution and from a fetal age of about 100 days the potentials had an amplitude of 200–400 μV . Such low amplitude values are typical for the adult animal (for sheep see Nougier 1963)

5 Repetitive stimulation

In several investigations it has been shown that the ability of evoked cortical responses to follow repetitive peripheral (Hunt and Goldring 1951 Scherrer and Oeconomos 1954 Grossman 1955 Molliver 1967 Myalivček 1968 Thairu 1971 Briquet and Verley 1972 Persson and Stenberg 1972) and central stimulation (Purpura 1961 a,b Scheibel and Scheibel 1964 1971 Grafstein 1963 Meyerson 1968 a,b, Conway Wright and Bradley 1969) increases significantly during development. In sheep fetuses younger than 70 days of age the tactile stimuli had to be given at intervals of 20–30 seconds to obtain reproducible cortical responses. In 75-day-old fetuses, the evoked cortical potential could follow a stimulus frequency of about 0.5 Hz, whereas the response followed a rate of about 1 Hz in fetuses older than 88 days. For comparison it may be mentioned that in nonanesthetized adult cats the recovery time of the primary somatosensory response has been reported to last 200 msec corresponding to a repetition rate of 5 Hz (Allison 1968)

In order to evaluate the susceptibility of the various response components to repetitive stimulation, experiments were performed in which the rate of stimulation was increased above that giving responses of constant amplitude. Fig 5 A and B shows a series of successive cortical potentials obtained at a stimulus rate of 1 Hz in a 65- and a 72-day-old fetus respectively. In the 65-day-old fetus the responses were reduced to a low-amplitude positive deflection followed by a slow negative wave (Fig 5 A). The corresponding

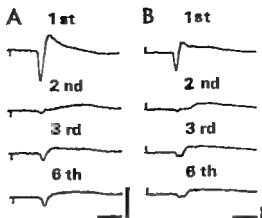


Fig. 5. Cortical responses to repetitive stimulation (1 Hz) in a 65-day fetus (A) and a 72-day fetus (B). Calibration: 100 μ V, 100 ms.

data (Fig. 5B) from the 72-day-old fetus show that the change in the cortical response was similar between the first and the second stimulation. However in the third response additional components appeared as represented by the second positive deflection and the negative hump superimposed on the slow negative wave (see also Fig. 19).

Facilitation was observed in a few fetuses between 80–120 days of age. In Fig. 6 is shown an example of such a facilitation in the response of an 82-day-old fetus induced by repetitive stimulation at a rate of 1 Hz. From the second stimulation there was a remarkable increase in amplitude of both the positive and the negative components. The facilitation appeared in a waxing and waning fashion and the negativity was split up into two peaks. Facilitation was never observed in younger fetuses. The alterations in the cortical response in older fetuses during high frequency stimulation were highly inconsistent.

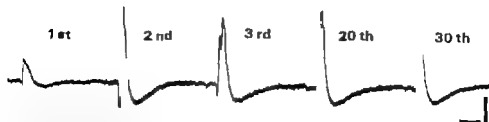


Fig. 6. Facilitation of the cortical response in an 82-day fetus by repetitive stimulation of 1 Hz. Calibration: 100 μ V, 200 ms.

6 Effect of strychnine

In an attempt to elucidate the development of inhibitory mechanisms in the cortex some experiments were undertaken in which the effect of strychnine on the evoked cortical potential was studied. In Fig 7 A is reproduced evoked responses in a 76-day-old fetus before and after topical application of 1% strychnine on the cortex. After strychnine there was a slight prolongation of the negative component. No significant change occurred in the response amplitude. Fig 7 B illustrates corresponding data from a 91 day-old fetus. After strychnine prominent changes could be observed in the response which displayed a twofold increase in the peak to-peak amplitude together with a marked prolongation of the duration. In addition a small initial positivity appeared and there was also a hump on the rising phase of the negativity. On the assumption that strychnine blocks inhibitory post synaptic mechanisms in the cortex (Pollen and Ajmone Marsan 1965 Stefans and Jasper 1965 Phillis and York 1967) the fact that strychnine only had an effect on the somesthetic response in older fetuses may be taken as evidence of a delayed maturation of cortical inhibitory synaptic systems during early periods of ontogeny

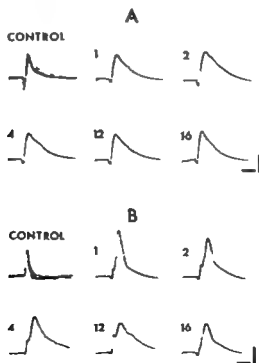


Fig 7 Effect of topical strychnine (1%) on the evoked cortical response in 76-day fetus (A) and 91-day fetus (B). Numbers above each record represent time in min after strychnine application. Calibration: 200 μ V, 100 ms.

7 Afterdischarges

In 6 older fetuses (aged 85—120 days) it was possible to evoke cortical afterdischarges in addition to the primary evoked potential (Fig 8 A—C). The afterdischarge usually consisted of an epoch of spindling activity superimposed on a negative d.c.-shift. The onset latency of the discharge amounted to 400—600 msec and it had a duration of 400—800 msec. The frequency of the waves in the afterdischarge varied from 8—16 Hz. The negative d.c.-shift was generally more pronounced in the older fetuses in which it could attain a value of about 400—600 μ V.

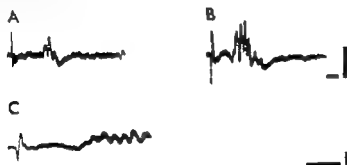


Fig 8. Cortical afterdischarges evoked by tactile stimulation of the trigeminal nose region. A, a 94-day fetus; B, 100-day fetus; C, a 105-day fetus. Calibration: 200 μ V, 200 ms.

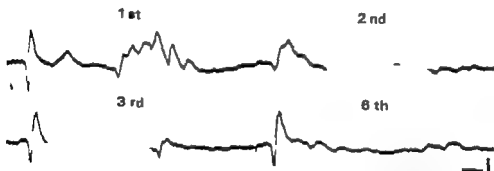


Fig 9. Selective effect of repetitive stimulation (0.5 Hz) on the 'primary' evoked response and the afterdischarge in 100-day fetus. Calibration: 100 μ V, 100 ms.

The cortical afterdischarge had a comparatively low resistance to repetitive stimulation even compared to that of the primary evoked response. As shown in Fig 9 the afterdischarge was almost completely abolished at a stimulation rate of 0.5 Hz, whereas the primary response was relatively unaffected. This finding may indicate that the two responses are generated by different sub-cortico-cortical mechanisms.

II. Development of somatosensory evoked field potentials

In order to evaluate the relative contribution of the activity in the different cortical layers to the building up of the evoked surface response during development, a series of experiments was performed in which the evoked potentials were recorded at different cortical depths. Because the development of the evoked surface response is characterized by a series of successive changes in the size of its positive and negative components, laminar potential analyses were based on the amplitudes of the evoked field potentials at the latencies corresponding to the peaks of the surface positivity and negativity. On the basis of the measured potential values, depth-potential profiles and estimates of the corresponding vertical current gradient were made (see Methods). The results to be presented comprise laminar analysis of the somatosensory evoked cortical potentials from five successive developmental stages each characterized by a specific pattern of the surface response.

62-day-old fetus

The results of the laminar potential analysis in a 62-day-old fetus exhibiting a surface positive cortical response are shown in Fig. 10. Upper traces in A represent the surface potential and lower traces illustrate corresponding field potentials recorded with a microelectrode at indicated depths. In recordings from depths less than $1000\ \mu$ the field potentials displayed a monopolar positive form, similar to the surface response. At greater depths the field potentials exhibited negative values with maximal amplitudes at a depth of about $2000\ \mu$. The peak latency of the deep negative wave was 115 msec which corresponded to that of the surface positivity. The cortical thickness amounted to $700\ \mu$ (horizontal dotted line in Fig. 10 B and C; see pp. 40). Thus the positivity of the surface response corresponds to positive field potentials located within the primitive cortex and to negativities located in the upper subcortical strata. The depth potential profile shows that the maximal negative values of the field potentials—corresponding to the peak of the surface positivity—are located in subcortical layers (Fig. 10 B).

The graph in Fig. 10 C illustrates the vertical current gradient derived from the depth potential profile. On the assumption that the negative maximum represents a net maximal current sink, this is located at a depth of about $1200\ \mu$. Similarly, if the positive maximum represents a net maximal source, this is situated at about $1000\ \mu$. To check the location of the microelectrode tips, marking experiments were performed with the Prussian blue method. The result revealed that the deep negativity and the net current sink were located in the subcortical strata (intermediate layer) of the immature telencephalic wall.

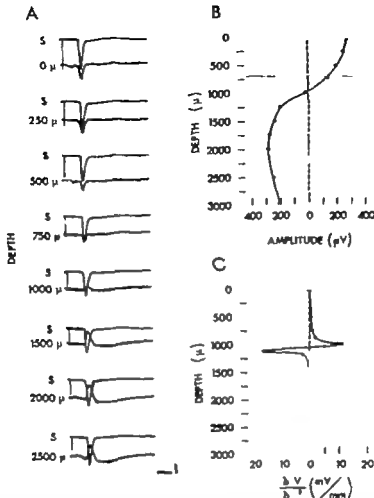


Fig. 10. Laminar analysis of evoked cortical potentials in a 62-day fetus. The upper records in A are surface potentials lower re field potentials recorded with a micro-electrode at indicated depths. Calibration 200 μ V 100 ms. Depth-potential profile (B) and estimate of the vertical current gradient (C) corresponding to the peak of the surface positivity. A positive value of the gradient curve indicates a source of vertical current and negative value indicates a relative sink (see Methods). The horizontal dotted lines (B and C) represent the approximate cortical thickness.

These observations indicate that early in development tactile stimulation of the trigeminal nose region does not activate neuronal elements within the cortex proper but evokes activity in strata immediate below the cortex.

70-day-old fetus

Laminar analysis of somatosensory evoked cortical potentials in a 70-day-old fetus is illustrated in Fig. 11. At this stage the surface response displayed

gradient graph were located at superficial ($350\ \mu$) and deep cortical strata ($1000\ \mu$; Fig 13 C). In between there was a high amplitude positivity in the vertical current gradient curve which may represent a midcortical net current source. The results obtained may indicate the existence of two preferential loci of neuronal activity corresponding to the cortical layers I—II and IV—V (see Fig 25 D).

110-day-old fetus

Fig 14 A shows the evoked cortical potentials from a 110-day-old fetus displaying a positive-negative surface response. The records of the field-potential show that during penetration the initial positive component dwindled quickly while the negative component grew and remained of high amplitude throughout the cortex. A shortening of the peak latency of the negative component was observed in superficial layers, and at a depth of $1000\ \mu$ it had a latency amounting to 70 msec, which corresponded to the peak of the surface positivity. The net maximal current sink (Fig 14 B, right) corresponding to the surface positivity was located in the midcortex at the approximate depth of the layer IV (see Fig 25 E). There was indication of two net current sources above and below this sink. The corresponding net maximal current sink to the surface negative wave was located in the uppermost part of the cortex (Fig 14 C right).

III Development of somatosensory evoked single unit activity

1 Response characteristics

Altogether 228 single unit responses were recorded in 37 sheep fetuses aged between 65 and 125 days. Samples of evoked single unit responses are shown in Fig 15—18 (lower records). In the fetuses younger than 68 days of age the unitary responses (9 of 12 units) generally consisted of a repetitive discharge displaying 2—4 spikes (Fig 15). The evoked unit activity recorded from the cortex of fetuses between 70 and 90 days was always a single discharge (Fig 16). Neither an increase of the intensity of the tactile stimulation nor an alteration of the stimulus locus on the nose caused a recruitment of additional spikes, contrary to what is found in the adult animal (Mountcastle 1957; Mountcastle, Davies and Berman 1957; Torre and Kennedy 1961).

Between 90—110 days of fetal age single spike responses were encountered from the majority of the neurons (Fig 17 96, left and 103). Occasionally repetitive discharges were observed (Fig 17 96 right and 103). At the same developmental stage a few neurons were seen to fire concomitantly with the cortical surface afterdischarge (Fig 24). During subsequent deve-

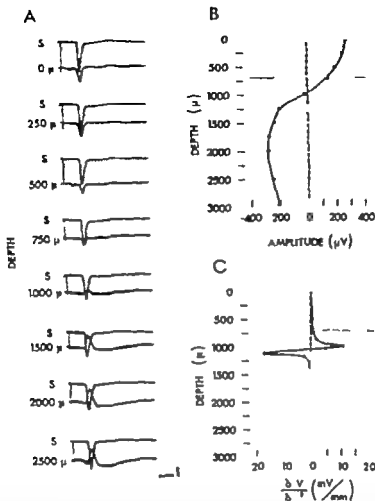


Fig. 10. Laminar analysis of evoked cortical potentials in a 62-day fetus. The pper records in A are surface potentials lower are field potentials recorded with a micro-electrode at indicated depths. Calibration 200 μ V 100 ms. Depth-potential profile (B) and estimate of the vertical current gradient (C) corresponding to the peak of the surface positivity. A positive value of the gradient curve indicates source of vertical current and negative value indicates a relative sink (see Methods). The horizontal dotted lines (B and C) represent the approximate cortical thickness.

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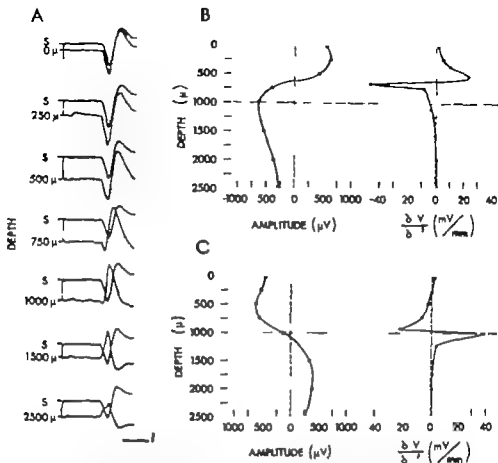


Fig. 11 Lamina analysis of evoked cortical potentials in a 70-day fetus. The upper records in A are surface potentials lower are field potentials recorded at indicated depths. Calibration 200 μ V 100 ms. B, depth-potential profile (left) and estimate of the vertical current gradient (right) at the peak of the surface positivity. C, the corresponding data at the peak of the surface negativity. The horizontal dotted lines (B and C) represent the approximate cortical thickness.

a biphasic positive-negative form with a predominance of the positive component (Fig. 11 A, upper traces). The configuration of the evoked field potentials (Fig. 11 A, lower traces) recorded at depths down to 250 μ was almost identical to the surface response. At increasing depths the field potentials displayed a gradual shift in wave form which could be characterized as a gradual shortening in the latency of the negative wave which eventually led to masking of the positivity at the depth of 1000–1500 μ . A maximal amplitude of the negative potential was met at a depth of 1000 μ . The shortest onset latency of the deep negativity was observed in responses obtained at depths of 1500–2500 μ and it appears from the records that the

these depths the field potentials have a monophasic negative configuration. In this developmental stage the lower border of the cortex was found to be situated at a depth of about $1000\ \mu$ (horizontal dotted line in Fig 11 B and C, see pp 40)

The graphs in Fig. 11 B show the depth potential profile (left) and the vertical current gradient (right) corresponding to the peak of the surface positivity. As seen the net maximal current sink was confined to the deep layers of the cortex. Negative values of the vertical current gradient curve correspond to depths of the primordia to cortical layers IV—VI (see Fig 25 B). Positive values of the gradient were obtained in superficial cortical layers. The depth-potential profile (Fig 11 C, left) and vertical current gradient (Fig 11 C, right) of the surface negativity reveal a predominantly deep intracortical location of the net current sink with its maximum at $950\ \mu$. The net current source was located at the lower border of the cortex proper

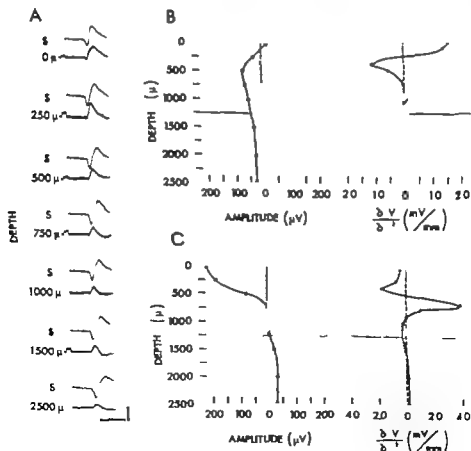


Fig. 12 Laminar analysis of evoked cortical potentials in an 80-day fetus. For further description see Fig. 11

These findings indicate that from 68–70 days of fetal life the neurons within the deeper layers of the cortex can be activated by the trigeminal afferent inflow

80-day-old fetus

The evoked field potentials in the cortex of an 80-day-old fetus displaying a surface positive-negative response with a predominating negativity show that with increasing depth the biphasic response was transformed into a monophasic negativity (Fig 12 A). This change in configuration was similar to that observed in the previous stage although it now took place in more superficial strata (down to 250 μ , Fig 12 A, lower traces). With increasing depths down to 2 500 μ the evoked field potentials remained negative. The depth-potential profile (Fig 12 B left) and estimate of the vertical current gradient (Fig 12 B right) of the peak of the surface positivity show negative

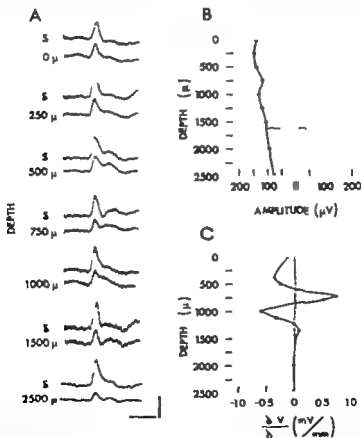


Fig. 13 Laminar analysis of evoked cortical potentials in 94-day fetus. Depth-potential profile (B) and estimate of the vertical current gradient (C) corresponding to the peak of the surface negativity. For further description, see Fig. 10

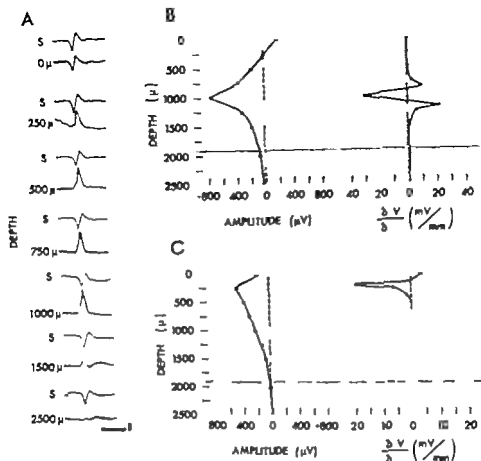


Fig. 14 Laminar analysis of evoked cortical potentials in 110-day fetus. For further description, see Fig. 11

maxima in the superficial cortical strata which later form the layers II—III (see Fig 25 C). The corresponding data for the negative wave (Fig 12 C) reveal net current sinks in the prospective layers I—III and in the deep strata of the cortex. The positive value of the vertical current gradient may correspond to a midcortical net current source. These results with prominent superficial current sinks are consistent with the assumption that the sensory stimulation now activated neurons throughout the cortex and that there is a predominance of activity in superficial strata.

94-day-old fetus

During the developmental period in which the evoked response had a monopolar negative form, the field potentials remained negative at all depth recordings (Fig 13 A and B). Two negative peaks in the vertical current

gradient graph were located at superficial ($350\ \mu$) and deep cortical strata ($1000\ \mu$ Fig 13 C) In between, there was a high amplitude positivity in the vertical current gradient curve which may represent a midcortical net current source. The results obtained may indicate the existence of two preferential loci of neuronal activity corresponding to the cortical layers I—II and IV—V (see Fig 25 D)

110-day-old fetus

Fig 14 A shows the evoked cortical potentials from a 110-day-old fetus displaying a positive-negative surface response The records of the field potential show that during penetration the initial positive component dwindled quickly while the negative component grew and remained of high amplitude throughout the cortex A shortening of the peak latency of the negative component was observed in superficial layers, and at a depth of $1000\ \mu$ it had a latency amounting to 70 msec, which corresponded to the peak of the surface positivity The net maximal current sink (Fig 14 B, right) corresponding to the surface positivity was located in the midcortex at the approximate depth of the layer IV (see Fig 25 E) There was indication of two net current sources above and below this sink The corresponding net maximal current sink to the surface negative wave was located in the uppermost part of the cortex (Fig 14 C, right)

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1 Response characteristics

Altogether 228 single unit responses were recorded in 32 sheep fetuses aged between 65 and 125 days. Samples of evoked single unit responses are shown in Fig 15—18 (lower records) In the fetuses younger than 68 days of age the unitary responses (9 of 12 units) generally consisted of a repetitive discharge displaying 2—4 spikes (Fig 15) The evoked unit activity recorded from the cortex of fetuses between 70 and 90 days was always a single discharge (Fig 16) Neither an increase of the intensity of the tactile stimulation nor an alteration of the stimulus locus on the nose caused a recruitment of additional spikes contrary to what is found in the adult animal (Mountcastle 1957 Mountcastle Davies and Berman 1957 Towe and Kennedy 1961)

Between 90—110 days of fetal age single spike responses were encountered from the majority of the neurons (Fig 17 96 left and 105) Occasionally repetitive discharges were observed (Fig 17 96 right and 103) At the same developmental stage a few neurons were seen to fire concomitantly with the cortical surface afterdischarge (Fig 24) During subsequent deve-

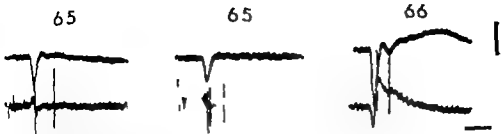


Fig. 15—18. Evoked surface and single unit responses in fetuses of various ages. The numbers above each record represent fetal ages in days. Calibration 200 μ V 100 ms.

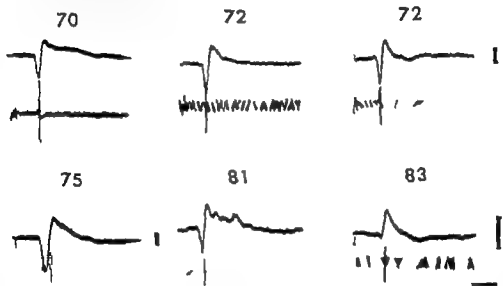


Fig. 16. Legend, see Fig. 15

development the relative number of evoked repetitive unit discharges increased. Thus, in a 115-day-old fetus most of the cortical neurons (12 of 17 units) responded with repetitive spikes (Fig. 18 right records). The train of discharge consisted of one to four spikes. Similar modal values of cortical neurons have been observed in the somesthetic cortex of the adult cat (Mountcastle *et al.* 1957). From the age of about 100 days, it was possible to establish a relationship between the relative site of the stimulation within the excitatory receptive field of the neuron and the intensity of stimulation on one hand and the pattern of discharge on the other. Thus, decreasing of the stimulation intensity or changing of the site of stimulation away from the center of the excitatory receptive field resulted in a reduction of the number of spikes per response and a lengthening of initial response latency. All these response properties have been found to be characteristic for the

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Altogether 228 single unit responses were recorded in 32 sheep fetuses aged between 65 and 125 days. Samples of evoked single unit responses are shown in Fig 15—18 (lower records) In the fetuses younger than 68 days of age the unitary responses (9 of 12 units) generally consisted of a repetitive discharge displaying 2—4 spikes (Fig 15) The evoked unit activity recorded from the cortex of fetuses between 70 and 90 days was always a single discharge (Fig 16) Neither an increase of the intensity of the tactile stimulation nor an alteration of the stimulus locus on the nose caused a recruitment of additional spikes, contrary to what is found in the adult animal (Mountcastle 1957 Mountcastle, Davies and Berman 1957 Towe and Kennedy 1961)

Between 90—110 days of fetal age single spike responses were encountered from the majority of the neurons (Fig 17 96 left and 105) Occasionally repetitive discharges were observed (Fig 17 96 right and 103) At the same developmental stage a few neurons were seen to fire concomitantly with the cortical surface afterdischarge (Fig. 24) During subsequent deve

frequency burst of activity (*cf* Huttenlocher 1961) from the high frequency discharge found in adu. (Huttenlocher 1961)

2 Repetitive stimulation and recovery cycles

In order to get a further idea of the development of the somatosensory system to transmit closely spaced stimuli, made in a few experiments of the effect of repetitive stimulation on the evoked unit activity in fetuses of various ages. The evoked unit responses were generally more resistant to repetition than the evoked surface potentials. The repetitive stimulation induced considerable alterations in the wave form of the potentials.

The responses depicted in Fig. 19 were obtained from a fetus stimulated with a frequency of 1 Hz. Except for the first evoked unit activity fired in response to stimulation. At higher frequencies the unit failed to follow and diverged. It further appears that the amplitude of the surface potential decreased during repetition and that it split towards splitting into two minute positive components concomitant unit activity from the third stimulus with the second of these positivities. An interpretation of the positive component of the positivity is of presynaptic and the negativity are of postsynaptic origin (*cf* Fig.

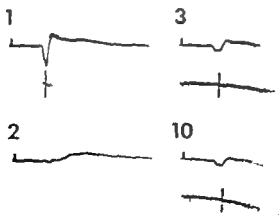


Fig. 19 Effect of repetitive stimulation (1 Hz) on evoked unit responses in 72-day fetus. Calibration 200 μ V 200 ms.



Fig. 20. Effect of paired stimulation on evoked surface and single unit responses in an 82-day fetus (A) with stimulus intervals of 400 msec (left) and 300 msec (right) and in a 105-day fetus (B) with stimulus intervals of 200 msec (left) and 100 msec (right). Arrows, time of stimulation. Calibration 200 μ V 200 msec.

examples of responses to paired stimulation in an 82-day-old fetus with a separation of the two stimuli amounting to 400 and 300 msec respectively. At stimulus intervals of 400 msec or more both stimuli caused a discharge. A similar experiment is illustrated in Fig. 20 B in a 105-day-old fetus and it appears that with intervals of 200 msec or more the second stimulation evoked a single unit response.

3 Relation to evoked surface responses

The time relation of single neuronal responses to the evoked surface potentials in fetuses of different ages can be observed in Fig. 15—18. In fetuses younger than 68 days, the initial spike of the evoked units discharged at a time corresponding to the peak of the surface positivity. In Fig. 21 is illustrated the latencies of the recorded units in relation to the evoked surface responses for four developmental stages. Each of these stages is characterized by a typical configuration of the surface response. It should be noted, however, that data from the initial developmental stage are not included since the number of units recorded was too small. The black histograms represent the latencies of the initial spike, whereas the dotted areas represent those of the second spike. In the developmental period

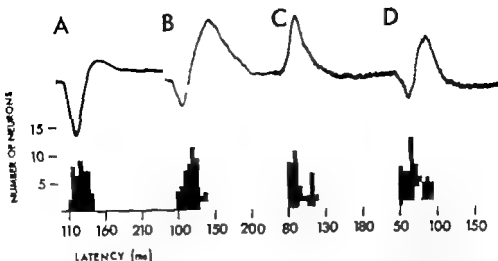


Fig. 21 Latencies of evoked single unit discharges in relation to the surface response in four developmental stages (A—D). Each of these stages is characterized by the configuration of the surface response. The black histograms represent the latencies of the initial spike, whereas the dotted areas represent those of the secondary spike. Note the data from the initial developmental stage are not included.

comprising fetuses of 68 to 75 days of age, all evoked single units discharged during the course of the positivity with a maximum at its peak (Fig 21 A). During the next stage (Fig 21 B) the single unit responses fired during the positivity and the initial phase of the negativity with a maximum at a latency value corresponding to the onset of the negativity. In these two stages the neuronal responses always consisted of a single spike.

The evoked units concomitant with the monopolar negative response occurred during the entire time course of the response with a preference of discharge during the initial phase (Fig 21 C). In fetuses older than 100 days characterized by a positive negative surface response the evoked units fired during the positivity and the initial phase of the negativity (Fig 21 D). The dotted histograms in Fig 21 C and D represent the latencies of the secondary spikes of the units with repetitive discharge found in fetuses older than 90 days.

4 Depth-distribution

In the fetuses less than 68 days of age it was not possible to record more than 12 units despite the fact that a large number of penetrations (about 100) were performed with microelectrodes of different recording characteristics. These 12 units were all encountered at depths of 1300—800 μ below the cortical surface. In order to check the depths from which these units were

negative d.c.-shift (*cf* Fig 8) It was possible to show that this afterdischarge may be accompanied by repetitive unitary responses (Fig 24) The left record shows a unit which discharged in conjunction with the primary response and during the afterdischarge. Another type of discharge more frequently encountered is illustrated in the right record where there was activity only during the cortical afterdischarge. No constant relation could be observed between the spikes and the individual waves of the afterdischarge.

IV Development of cortical morphology

Some data on the early morphological development of the neocortex in fetal sheep are presented in this section to facilitate the understanding of the relationship between structure and function. A great deal of information has been collected in an extensive investigation by Åström (1967) In the present study some complementary results will be presented with special regard to the maturation of the cortical stratification. Such data are of particular significance in relating the neuronal events to the cortical structure.

In Fig 25 is depicted a series of brain sections stained with cresyl violet, from fetuses of various ages. The specimens were taken from the cortical areas in which somesthetic evoked responses were recorded. In the older fetuses with gyrencephalic brains these areas correspond to the area pre-parietalis of the adult animal (Rose 1942)

During the early developmental period when the fetal brain is lissencephalic, the neopallial wall is made up of three distinct concentric strata: the cortical plate, the intermediate zone and the germinal layer (Åström 1967). The immature cortex proper consists of two layers, the marginal and pyramidal layers, which successively develop into the 6-layered adult neocortex. In the brain section from a 62-day-old fetus (Fig 25A) the cortex proper and the upper subzone of the intermediate layer can be seen. This type of stratification is characteristic for an early phase of neocortical ontogeny. The lightly stained marginal layer (a) contains horizontally orientated cells, the Retzius-Cajal cells (Retzius 1891, 1893; Cajal 1960; Åström 1967). An incipient stratification can be observed in the pyramidal layer (b—d) with an indication of a light band in its middle third (c). The cells in the pyramidal layer have an immature appearance. In the outer part small dark cells of bipolar shape are arranged in compact vertical columns; in the inner layers a widening of the intercellular spaces can be observed. The subpyramidal layer (e) contains some large cells with a relative mature appearance, presumably stellate cells (*cf* Åström 1967).

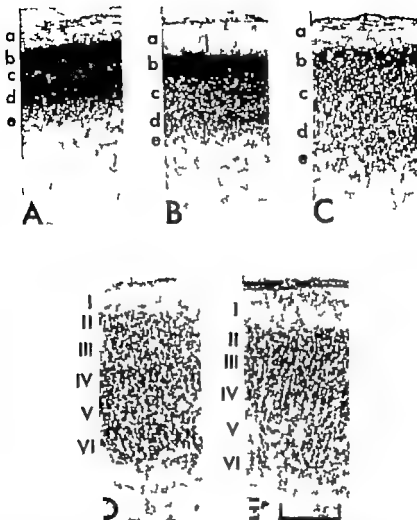


Fig. 25 Somesthetic cortex at different stages of maturation. Brain sections from *f* tissues of 62 (A) 69 (B) 82 (C) 98 (D) and 127 days of age (E) Cresyl violet stain. Horizontal bar represents 500 μ .

The lamination of the cortex in a 69-day-old fetus stands out more clearly and consists of 5 identifiable layers (Fig 25 B) The uppermost part (b) of the pyramidal layer is crowded with cells of the same appearance as in the younger animals. Compared to earlier stages the light band in the middle of the pyramidal layer (c) has become wider and more prominent. This is probably due to the fact that the evolution of dendritic and fibrillar processes has caused a separation of cells and consequently a widening of the intercellular spaces (Lorente de N6 1933 Åström 1967) The pyramids

of the deep dark layer (d) are less packed and more mature than those in the superficial pyramidal layers (b). Large stellate cells can be observed for the first time within the inferior pyramidal layer. These cells are the largest neurons in the neocortex at this developmental stage—a fact which may be of functional significance in the present context. The cellular morphology in this stage has been described by Åström (1967) in detail with the aid of the Golgi method.

The section (Fig. 25 C) from a 82-day-old fetus represents the next developmental stage and a more definite 5-layered stratification is now discernable. In the Nissl preparations most cells appear to have reached a comparatively advanced stage of development. However the most superficial cells are still immature and form a thin dark stratum (b) below the marginal layer (a).

A clear 6-layered stratification similar to what is found in the adult animal (Rose 1942) is observed in the cortex of a 98- and a 127-day-old fetus (Fig. 25 D and E). Mature pyramidal cells are found in all cortical layers even in the most superficial ones.

To summarize the cortical cells and strata, as studied in Nissl stained preparations become differentiated in a definite sequence. The marginal and the subpyramidal layers are the first to become differentiated. These strata will form the layer I and the lower part of layer VI in the adult cortex. Thereafter the lightly stained stratum of the pyramidal layer develops and will become the layers IV and lower III. Parallel with these changes, stellate cells are incorporated in the lower pyramidal layers. The deep pyramids which constitute layers V and VI will then attain their mature appearance. During the subsequent development the cells within layers III and II differentiate to their mature form (*cf.* Åström 1967).

For the interpretation of the results in the current investigation the reliability of the estimations of the depth locations of neuronal activity as well as its morphological substrate are of paramount importance. Therefore it is necessary to know the degree of shrinkage induced by the histological preparation. For this purpose an iron deposit was produced through a micro-electrode tip at a fixed cortical depth as read off by the micrometer during an actual experiment (*e.g.* Ånggård 1965). By comparing the depths of the Prussian blue spots in brain sections with the corresponding micrometer readings the shrinkage factor could be determined. In the present fetal material the shrinkage of the neocortex amounted to about 30% (range 25–35%) which is somewhat larger than the corresponding value (20–25%) reported for the adult rat cortex (Borbély 1970). This difference may be explained by the fact that the water content of the immature brain is higher

than that of the mature brain (e.g. Vernadakis and Woodbury 1962). In Fig 26 A is shown an example of an iron deposit produced at a depth of $500\ \mu$ in the neocortex of a 71-day-old fetus contraststained with cresyl violet. This deposit was located at the border between the immature superficial dark (prospective layers II and III a cf Fig 25 B layer b) and the relatively more mature middle light layers (prospective layers III b and IV cf Fig 25 B, layer c)

The markings shown in Fig 26 B are from an 82-day-old fetus. The whole track down to $500\ \mu$ was marked and the lower limit corresponded to the superficial cortical strata (layers II and III cf Fig 25 C layer b). The deposit at $1000\ \mu$ was located at the lower border of the prospective layer IV (cf Fig 25 C, layer c). On the basis of the shrinkage factor of 30% the approximate cortical thickness was determined from the cresyl violet stained brain sections made at different developmental stages: $700\ \mu$ at the age of 52, $1000\ \mu$ at 72, $1250\ \mu$ at 82, $1650\ \mu$ at 98 and $1900\ \mu$ at 127 days. These values have been used to denote the cortical thickness in Fig 10 11 12 13 14 and 22.

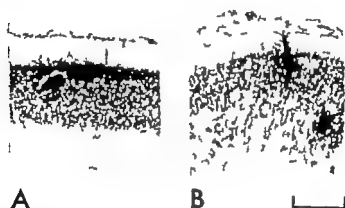


Fig. 26 Prussian blue markings in cortical sections stained with cresyl violet. A, at a depth of $500\ \mu$ in 71-day fetus; B, at depths of $500\ \mu$ and $1000\ \mu$ in an 82-days fetus. Horizontal bar represents $500\ \mu$.

DISCUSSION

The present results demonstrate that a somesthetic response to tactile stimulation can be recorded from the cortical surface of sheep fetuses already at a very early stage of ontogeny thus confirming the original study by Molliver (1967). During the subsequent development the response undergoes a sequence of characteristic alterations. Earlier investigations on cortical potentials evoked by somesthetic stimulation in dogs, cats, rabbits and rats during the postnatal period show that a response is present at birth (Scherer and Oeconomos 1954 Grossman 1955 Marty 1962 Delhaye-Bouchaud 1964 Thauru 1971 Verley and Rokyta 1972). A comparison of the functional characteristics of the cortical response in these altricial postnatal animals and in the fetal sheep of different ages suggests that the developmental stage of the somatic sensory system of newborn dogs, cats, rabbits and rats corresponds approximately to that of the 80—90-day-old fetal sheep. In addition, the sequence of changes in the response, in particular with regard to its configuration during the postnatal period of these animals is very similar to those observed in the later prenatal stages of sheep. These observations lend support to the notion that the difference in rate of pre- and postnatal functional development of these species (Adolph 1970) also applies to the somatosensory system (*cf.* Gottlieb 1971). Thus, the present study on fetal sheep covers the initial periods of development of the somatosensory system, whereas earlier investigations on postnatal animals have been confined to relatively more mature stages of ontogeny.

1 Aspects of the mode of operation in the immature somatosensory system

Unitary recording in the adult cortex is subject to bias (Towe and Harding 1970). This bias may be even more important in the case of immature neurons, which are small and have fragile membranes as deduced from their structural and functional properties different from those found in the adult (*cf.* Purpura 1972). As a matter of fact, stable recordings during long lasting sessions are very difficult to obtain and impossible to achieve in the early prenatal cortical stages. Nevertheless, a large number of penetrations were made into the subcortex at each developmental stage with micro-

electrodes of different recording characteristics. It should be recalled that general anesthesia shown to decrease responsiveness of cortical neurons (Mountcastle *et al.* 1957) has not been used. It cannot be excluded that factors at the stimulation site e.g. changes in the tissue properties and size of the stimulated nose area in the relation to the stimulating probe may influence results of the present study such as the development of amplitude and cortical distribution of the response. However there are reasons to believe that these factors do not contribute significantly and in a predictable way to the parameters of development observed. Thus, for instance, the response amplitude shows both an increase and a decrease during the period of development when the relative stimulation area decreases.

The functional significance of the sensory evoked repetitive discharge in the subcortical units of the young fetal sheep is unknown. A similar pattern of discharge of subcortical neurons evoked by light stimulation has been observed in the newborn kitten (Huttenlocher 1967). When, during development evoked units were first obtained within the cortex they responded with a single spike. Such a discharge pattern has also been demonstrated in experiments on newborn kittens and shown to represent postsynaptic activation (Purpura *et al.* 1965). Another aspect of the low level of responsiveness of the immature cortical neurons is their characteristic paucity of spontaneous discharge (Persson, in preparation *cf.* Hyvärinen 1966, Huttenlocher 1967, Laget, Thomson and Delhay-Bouchaud 1967, Armstrong-James 1970, Rubel 1971). Contrary to what has been found in the adult animal (Mountcastle 1957, Mountcastle *et al.* 1957, Towe and Kennedy 1961, Diamond-Smith 1966) the immature cortical neuron responds only with a single spike regardless of the strength and location of the stimulus within the peripheral receptive field. This finding points to a fundamental difference in the function of the immature and the mature somatosensory system to code information about the stimulus parameters. The appearance during development of cortical neurons capable of evoked repetitive discharge denotes the transition to a stage when the somesthetic system begins to operate in a way qualitatively similar to that of the adult. It is important to recall that this maturational process is highly dependent on extracortical functions such as an increased maturity of the tactile cutaneous receptors and of the peripheral sensory nerves (Elkholm 1967, Kasprzak, Tapper and Craig 1970).

Contrary to the findings in a 76-day-old fetus, the clear-cut releasing effect of strychnine on the evoked potential in a 90-day-old fetus as well as the presence of cortical units at a comparable developmental stage, whose spontaneous discharge was arrested by peripheral stimulation, strongly suggest that inhibitory mechanisms within the cortex are effective at that later stage

of development. In corresponding developmental periods of postnatal kittens, pronounced long lasting IPSPs have been observed in cortical neurons as a result of electrical stimulation in the thalamus (Purpura *et al.* 1965). Furthermore, the duration of these IPSPs is of the same order of magnitude as the duration of the inhibitory arrest of spontaneous units observed in the present study. These findings lend support to the idea that there may be a relative delay in the maturation of cortical inhibitory mechanisms (*cf.* Huttenlocher 1967, Meyerson and Persson 1973). The late development of axosomatic synapses, considered inhibitory in nature (*e.g.* Colonnier 1968) compared to axodendritic ones (Voeller *et al.* 1963, Møller *et al.* 1968, Adinolfi 1971, 1972) may serve as further evidence for such a supposition. However the significance of axodendritic inhibition in the immature cortex (Purpura 1971) and hippocampus (Purpura, Prelevic and Santini 1968) has been proposed recently. It has been claimed that inhibitory postsynaptic mechanisms, both in the cortex and hippocampus show a precocious development compared to excitatory ones (Purpura 1969, 1971, 1972). The fact that these observations were made on newborn kittens (compare with fetal sheep of 80–90 days of age) implies that no stage was studied sufficient immature to justify a general statement about the relative rate of functional development of excitatory and inhibitory postsynaptic mechanisms in the cortex. Intracellular recordings of the responses in cortical neurons to peripheral stimulation have to be performed in fetal animals to settle this question.

In recent publications (Scherrer *et al.* 1968, 1970) it has been discussed that the time velocity and flow of neuronal signals are important factors, which contribute to the differences in function of neonatal and mature nervous system. The over-all conduction time from the receptors to the cortex of very young sheep fetuses is long. Between 80 and 120 days, there is a rapid and continuous decrease in conduction time. This change is dependent upon several factors such as changes in synaptic transmission efficiency increasing of conduction velocity and lengthening of conduction pathways during growth (*cf.* Mysliveček 1968 a). The major factor contributing to the decrease of the onset latency is presumably the increase of afferent fiber diameters and in particular the development of myelin as repeatedly emphasized (references, see Skoglund 1969). In the fetal sheep myelin can be detected in the trigeminal nerve at 60 days of age (Barlow 1969) in the main sensory trigeminal nuclei at 66 days (Romanes 1947) and in the thalamic radiation at 78 days (Barlow 1969). Conduction time approaching adult values was obtained in fetuses of 120 days of age. The fact, that the distance from skin receptors to cortex in these fetuses is short compared to that of the adult animal, implies that the conduction time attains adult

values long before conduction velocity (*cf* Scherrer *et al* 1968) This finding is in accordance with the wellknown fact that the conduction velocities of peripheral (Huxh 1939 Skoglund 1960) and central nerve fibers (Verley 1967 a, Meyerson 1968 b Conway *et al.* 1969) of fetal sheep and of neonatal cats and rabbits are slow The fatiguability of the somatic sensory system is extremely marked in young fetuses, as a stimulus interval of up to 20 sec had to be used to obtain reproducible responses with constant amplitudes During development there is an increased capacity of the system to mediate closely spaced signals and generate constant electrocortical responses (*cf* Scherrer and Oeconomos 1954 Molliver 1967) Thus, in the fetal sheep one is confronted with an ineffective somesthetic system in terms of low conduction velocities and reduced flow of the sensory neuronal signals which are considered to be of importance for the sensory feed-back mechanisms and memory processes (Scherrer *et al* 1968 1970)

2 Development of the specific and nonspecific somatosensory systems

The similarity between the somatotopic organization of the ipsilateral upper lip in the immature fetal sheep and in the adult (Adrian 1943 Hatton and Rubel 1967) indicates that the lay out of adult cortical projection is present already at an early developmental stage. The results from a recent study on the cortical somatotopy of newborn and adult cats (Rubel 1971) are in accordance with this finding, since it was found that the projections from all the contralateral body surface to the primary sensorimotor cortex were present at birth and organized in a similar way to that of the adult animal. From the time when the adult pattern of convolutions and fissures can be observed in the fetal sheep the ipsilateral evoked potentials were found to be confined to those areas which correspond to the primary and secondary somesthetic areas in the adult as defined electrophysiologically (Adrian 1943 Woolsey and Fairman 1946) and morphologically (Rose 1942) In no case was it possible to record somesthetic responses from other brain areas The predominately negative evoked response of fetuses between 80—90 days of age is identical to the cortical potential obtained in newborn cat (Purpura 1961 a,b 1962) and rabbit (Verley *et al* 1966 Verley 1967 a, Verley and Siou 1967) in response to electrical stimulation of the specific thalamic relay nuclei In the adult sheep it has been shown that electrical stimulation applied to the sensory endings of the trigeminal nerve evokes prominent responses in the ipsilateral ventroposteromedial (VPM) nucleus (Richard, Auffray and Albe Fessard 1967 Cabral and Johnson 1971) as well as in the primary somesthetic cortex (Nougier 1963) In view of these findings, it is assumed that the cortical response evoked by tactile stimulation of the

ipsilateral trigeminal nose region in sheep fetuses during development results essentially from an activation via the specific thalamocortical system. A similar conclusion about the trigeminal afferent input to the somatic sensory area of newborn rabbit has been reported (Verley 1967 b)

It is a wellknown fact that in the adult unanesthetized animal different types of sensory stimuli may evoke responses within the so-called association areas of the cortex (e.g. Buser and Bignall 1967 Thompson, Bettinger Burch and Groves 1969). The failure to record such responses to trigeminal tactile stimulation in any of the fetuses indicates a relative retardation of this associative somatosensory system. It is of interest in this connection to refer to a recent study by Mayers, Robertson Rubel and Thompson (1971). They demonstrated that in postnatal kittens, sensory evoked unit responses in association areas developed comparatively late and that the somatosensory units appeared later than visual and auditory ones.

A cortical afterdischarge triggered by single sensory stimulation has been observed in the somesthetic (Scherrer and Oeconomos 1954) and visual cortex (Marty 1962 Huttenlocher 1967 Rose *et al* 1972) of unanesthetized postnatal kittens. The findings in the present study that the cortical after discharge appeared later in development than the primary evoked response, and that it was selectively suppressed by low frequency repetitive stimulation support the view that these two cortical activities are generated by different subcortico-cortical mechanisms. Furthermore it was frequently observed that cortical neurons fired solely concomitantly with either of the two electrocortical activities. There is reason to believe that the appearance during development of the cortical afterdischarge is dependent on the functional maturation of nonspecific reticular and thalamic structures and their integration with the afferent specific system. Firstly the simultaneous appearance and the similar pattern of the evoked cortical afterdischarge and spontaneous EEG-spindles (Bernhard *et al* 1959 Bernhard and Meyerson 1968 Meyerson 1968 b) may indicate a common subcortical drive for these electrocortical activities. The importance of intrathalamic integration for the synchronization of the thalamocortical rhythmic activities in the adult (references, see Andersen and Anderson 1968 Purpura 1968 1972) and immature animal (Thatcher and Purpura 1972) has been stressed. Secondly since the flash-evoked cortical afterdischarge observed in postnatal kittens develops into a desynchronizing activity typical of adult electrocortical arousal it has been considered to constitute a primitive reticular arousal (Rose *et al* 1972).

On the basis of the foregoing discussion, it seems likely that there is a differential development of specific and nonspecific afferent systems projecting to the somatosensory cortical areas from the trigeminal region. The

specific pathways may activate the cerebral cortex already during early prenatal stages as shown by the presence of the primary evoked response whereas generalized and associative activation involving the ascending reticular system integrated with the specific afferent system, seems to appear comparatively late during ontogeny

In this context it is of relevance to make a comparison with the differential development of the specific and nonspecific components of the visual and auditory systems. It has recently been demonstrated in the prenatal sheep that the development of visually evoked cortical responses occurs in two phases (Pernon and Stenberg 1972). A similar developmental course has previously been described in the postnatal development of cat, dog and rat (Marty *et al.* 1959, Marty 1962, Fox 1968, Myllyveček 1968 a, Rose 1968 a,b 1971, Rose and Lindsay 1968). Based on the different reactions to selective subcortical lesions of the different components of the cortical response it has been proposed that the early appearing negative component is mediated via the nonspecific optic projection. Components appearing later in development have been considered to be dependent on the specific visual pathways (Rose and Lindsay 1968). On the basis of studies on auditory evoked electrocortical responses in postnatal dogs to electrical stimulation in the periphery and in the medial geniculate body it has been assumed that the specific thalamic relay nucleus is indispensable for evoked auditory responses in the newborn animal (Myllyveček 1968 b).

3 Functional development of the somatosensory cortex and its morphological correlates

Earlier investigations on the postnatal development of evoked surface responses in sensory cortex in various species have demonstrated the presence of a predominant surface-negative potential immediately after birth. The precocity of the superficial axodendritic neuropile has been considered to account for this feature of the evoked potential (Marty *et al.* 1961, Scheibel 1962, Marty and Scherrer 1964, Purpura *et al.* 1964). However the characteristic surface positive wave form of the somatosensory evoked response in immature fetal sheep suggests a different mode of electrocortical activation in the cortex during early prenatal stages of ontogeny. This is not due to species-specific organization of the sheep's cortex, since it has been reported that predominantly positive evoked cortical responses may be obtained also in the cortex of fetal dog (Molliver personal communication). In addition, somatosensory evoked cortical responses displaying initial positivity have occasionally been recorded in newborn rats (Thalru 1971).

The results of the laminar recordings of both gross and unit evoked activity

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On the basis of the foregoing discussion, it seems likely that there is a differential development of specific and nonspecific afferent systems projecting to the somatosensory cortical areas from the trigeminal region. The

since its net current sink is located in the primordium of layer IV and since the concomitant single unit activity is encountered at the same cortical depths. A small fraction of the surface positivity might be of a presynaptic origin as shown by its ability to follow repetitive stimulation. It has been assumed that superficial inhibitory cortical activation contributes to positive surface potentials in the adult cortex (Purpura 1959 Creutzfeldt *et al* 1966 a). It is less likely that in young sheep fetuses superficial inhibition substantially contributes to the positive component of the surface response since topically applied strychnine has no effect.

At the same developmental stage the negative component of the evoked surface response corresponds to a deep current sink-source pair at the lower border of the cortex but no concomitant single unit activity can be recorded. A subthreshold excitatory drive on the deep cortical neurons without spike generation may be suggested, since it has been assumed that immature cortical neurons exhibit a relatively high level of postsynaptic excitability but a strikingly low level of spike responsiveness (Purpura 1972). However the possibility of an inhibitory activation of neuronal elements situated at the lower cortical border cannot be disregarded. There is no experimental evidence of a synaptic activation of neurons in the superficial cortical strata.

Histological investigations of the cortex of fetal sheep during this developmental stage demonstrates the presence of fairly well-defined lamination appearing as 5 layers of alternating high and low cell density (*cf* Åström 1967). Layers with low cell density have been considered to be caused by separation of neurons due to the formation of dendrites and axons (Lorente de Nó 1933 Åström 1967). There is a good correlation between the cortical depths from which evoked activity was obtained and the depths of the low cell density layers, which presumably contain the morphological pre- and postsynaptic substrate for the activity.

The afferent fibers reaching the cortex at this developmental stage are directed towards the marginal and midcortical layers (Åström 1967). In recent studies on the fetal cat brain during a comparable developmental stage it has been shown that a new set of afferent fibers, assumed to be specific, penetrates into the lower region of the pyramidal layer (Marín Padilla 1971 1972). The apical dendrites with signs of developing shaftspines and the well-developed basal dendrites of the deep pyramidal neurons and the mature stellate cells, present in deep cortical layers (IV—VI Åström 1967) may serve as postsynaptic elements. Of special interest is the appearance of spines on the apical shafts of the deep pyramids since they have been considered to constitute a main postsynaptic locus for the afferent fibers of the specific thalamic nuclei to the somesthetic (Jones and Powell 1970) as well as to the

visual cortex of the adult animal (Globus and Scheibel 1967 a,b Garey and Powell 1971) In a recent electron microscopic study of the vertical distribution of synapses in the neocortex of newborn dog (Molliver and van der Loos 1970) three regions of high synaptic density were found one corresponding to the marginal layer and two corresponding to the deep layers containing the large pyramidal neurons. On the basis of the functional data presented by Molliver it was assumed that synaptic functions were first established in the deep cortical strata. This idea harmonizes with the results of the present study It should be added that the evolvement in deep layers of recurrent axonal collaterals and stellate cells in the cortex of fetal sheep may provide the morphological substrate for intracortical connections. The functional role of stellate cells, which in the mature neocortex amount to 25% of total population of nerve cells, has been emphasized (Scheibel 1962 Schädé Backer and Colon 1964)

During the following developmental period (75—90 days) in fetal sheep there is a transformation of the surface response from a biphasic positive negative to a monophasic negative form. This alteration is combined with an increasing amplitude and decreasing latency of the negative component which thus seems to mask and eventually extinguish the initial positive wave. As mentioned earlier the monopolar negative response in the fetal sheep is identical to that obtained in neonatal altricial animals with peripheral somesthetic stimulation (e.g. Scherrer and Oeconomos 1954 Marty 1962 Thairu 1971) The observations of net current sinks corresponding to the surface positivity and negativity in cortical layers I—III suggest the presence of superficial excitatory generators. This interpretation is substantiated by the presence of evoked unit activity in these same layers. In addition the laminar potential data suggest that the surface response receives a greater contribution from the neuronal activity in superficial cortical layers than from the activity in the deeper layers. In recent studies on sensory evoked responses in the somesthetic cortex of newborn rabbits (Verley and Rokyta 1972) and in the auditory cortex of postnatal cats (König, Pujol and Marty 1972) similar results have been reported The observations that during this developmental stage strychnine alters the form and amplitude of the evoked surface response and that peripheral stimulation may inhibit spontaneously active cortical neurons lend support to the view that inhibitory phenomena may play an important role in the cortex. This notion is compatible with the demonstration of prominent and prolonged IPSP's in cortical (Purpura *et al* 1965) and hippocampal neurons of newborn cats (Purpura *et al* 1968)

There are several morphological characteristics which may account for the data indicating a functional maturation of the superficial layers of the

neocortex during this developmental period (75—90 days). Thus, the immature bipolar cortical neurons in layers II—III attain their mature form (Åström 1967). The successive evolution of apical and in particular basilar dendrites of the pyramids in these layers, indicates that this stage constitutes the formative phase of the postsynaptic elements in the superficial neuropile. Electron microscopic investigations of the neocortex of fetal and newborn cat have shown a characteristic abundance of axodendritic synapses in the superficial strata at a corresponding developmental stage (Voeller *et al.* 1963; Adinolfi 1971, 1972). Furthermore, in a synapto-architectonic study on the somesthetic cortex of the newborn dog a high level of synaptic density was observed in the marginal layer containing the Retzius-Cajal cells and the terminal arborizations of the apical dendrites (Molliver and van der Loos 1970). A spread of neuronal activity can be mediated from deep to superficial strata by the way of recurrent collaterals of the deep pyramids and interneurons. The presence of elaborate interneurons is of particular interest since cells of this class (*cellule à double bouquet dendritique de Cajal* Cajal 1935) have been assumed to be responsible for the vertical spread of excitation in the vertical columns of the adult cortex (Colonnier 1964). In view of observations made in fetal and newborn cat (Marín-Padilla 1972; Læmle, Benhamida and Purpura 1972) one may also assume that in fetal sheep displaying surface negative responses, afferent fibers directly activate the superficial layers.

In the present investigation the results obtained from the laminar analysis of evoked field potentials and from the vertical distribution of evoked unit discharges reveal that during the early prenatal ontogenesis of the sheep the trigeminal afferent inflow initially activates neuronal elements in the immediate subcortical stratum. During subsequent development the activation takes place in the deep layers of the cortex and successively invades superficial layers. This corticopetal gradient of functional development is expressed by the gradual alterations in the configuration of the evoked response from a surface positivity to a surface negativity. It is relevant to refer to findings on the developing visual (Persson and Stenberg 1972) and transcallosal cortical responses (Meyerson 1968 b) in fetal sheep. At their earliest appearance these responses display a predominating surface positive wave and subsequently undergo changes similar to those of the somesthetic response. These observations suggest that, in general, the functional development of the cortex takes place along a corticopetal gradient. The morphological correlation to this pattern of functional development is the well documented observation that, in general, the maturation of the neurofibrillar organization in the cortex of mammals, takes place along a corticopetal gradient from

deep to superficial strata (Vignal 1888 Lorente de N6 1933 Cajal 1960 Angevine and Sidman 1961 Berry and Rogers 1963 Caley and Maxwell 1968 for discussion, see Molliver and van der Loos 1970)

The beginning of the final developmental period is marked by the reappearance of a small initial positivity in the surface response. Later the positivity increases in amplitude and the response attains the adult positive-negative configuration. The reappearance of the positive component during late prenatal development is paralleled by marked changes of the temporal relationship of the net current sources and sinks. The deep sink observed in previous stages is now recorded at an earlier phase of the cortical activation and this in turn gives rise to the initial surface positivity. Thus, the well-known transformation during development of the evoked cortical potentials from a surface negativity to a surface positive-negative form may tentatively be interpreted as being the result of an unmasking of a pre-existing excitation located in the lower part of the cortex. It is interesting to note that the deep and superficial centers of neuronal activity generating the positivity and negativity of the developing cortical response in fetal sheep resemble the A and B-generators described in adult cats (Calvet, Calvet and Scherrer 1964 Calvet and Calvet 1965 Calvet, Calvet and Langlois 1965) and in newborn rabbits (Garma and Verley 1966 1967 Verley 1965 1968). On the basis of the results described one may assume an early net excitatory activation of neurons in the midcortex followed by the spread of activity to superficial strata much in the same way as has been suggested for the adult somesthetic cortex (Eccles 1964 Towe 1966 Landau 1967)

From studies in postnatal animals it has been proposed (Scheibel 1962 Scheibel and Scheibel 1964 1971) that the evolvment of the neuropile in layer IV with regard to specific corticopetal fibers and stellate cells may account for the reappearance of the surface positive wave. Alternatively the significance of a further development of basal dendrites of the deep-seated pyramids together with the development of axosomatic synapses has been emphasized (Marty *et al* 1961 Marty 1962 Marty and Scherrer 1964 Purpura *et al* 1964). The details of the later morphological maturation of the neocortex of fetal sheep are still lacking. However well developed basal dendrites in conjunction with the deep pyramids are present in fetal sheep at an earlier stage, and it is therefore likely that the development of the neuropile of the layer IV constitutes a major factor for the reappearance of the surface positivity.

SUMMARY

The prenatal development of somatosensory cortical functions was investigated by recording evoked surface and depth gross responses as well as extracellular single unit activity following tactile stimulation of the trigeminal nose area. Experiments were performed on externalized and nonanesthetized sheep fetuses with ages from 42 days to full term kept in contact with the decerebrate ewe through the intact umbilical cord.

Lammar analyses of evoked field potentials and depth-distributions of evoked single unit responses showed that with age there was a change of the locus of activity in the somesthetic cortex which reflected alterations in the configuration of the surface response. The functional maturation of the somatosensory system was further denoted by changes of the evoked surface potential with respect to its cortical distribution latency amplitude and ability to follow repetitive stimulation occurring as a function of age.

During the developmental period between 42—68 days the evoked surface response consisted of a long latency positive wave. Negative evoked field potentials and evoked single unit responses were obtained only in the sub-cortical strata of the telencephalic wall. Whether or not this single unit activity is of pre- or postsynaptic origin cannot be definitely stated. No signs of intracortical activation could be observed. Thus, the results indicate that during early ontogeny the trigeminal somatosensory system operates as a dead-end afferent system with no functional contacts with the cortex proper.

During the developmental stage between 68—75 days the evoked surface response had a biphasic positive-negative configuration with a predominating positivity. Both the lammar analysis of the evoked potentials as well as the depth-distribution of the evoked single unit activity showed that around 68—70 days the tactile stimulation activated the cortex for the first time. The evoked cortical activity was present in the primordia of cortical layers IV—VI and there were no signs of activation of superficial strata. The cortical neurons responded with a single spike regardless of the strength and of the location of the stimulus within the peripheral receptive field. Correlative morphological data indicate that afferent fibers shown to reach the mid pyramidal layers may constitute the presynaptic substrate for the cortical

activation. The dendrites of the deep pyramids and the mature stellate cells, present in the deep cortical layers (IV—VI) may serve as postsynaptic elements.

During the subsequent developmental period (75—90 days) the surface response underwent alterations in configuration from a predominantly positive to a monopolar negative form. The surface negative response corresponded to negative field potentials obtained in all cortical layers. Unit responses from neurons activated by the trigeminal inflow could now be recorded throughout the cortex. Correlative morphological data indicate that this stage constitutes the formative phase of the postsynaptic structures in the superficial neuropile. A spread of neuronal activity from deep to superficial strata may be mediated by way of intracortical connections through interneurons and recurrent collaterals of the deep pyramids as well as of corticopetal afferent fibers directed towards the superficial neuropile.

The laminar analysis of evoked field potentials and the vertical distribution of responding units show that at the end of the first trimester the trigeminal inflow activates neuronal elements in the immediate subcortical stratum. During the following development the activation takes place in deep layers of the cortex and later on also invades more superficial strata. This corticopetal gradient of functional development is expressed by the gradual alterations in the configuration of the evoked surface response from a positivity to a negativity.

On the basis of the selective effects of topical strychnine on the developing somesthetic surface response it is assumed that there is a delayed development of inhibitory postsynaptic mechanisms in the cortex. Arrest of spontaneously discharging cortical units as a result of trigeminal stimulation was not possible until a fetal age of about 90 days.

The representation of the ipsilateral upper lip in the cortex of immature fetal sheep was similar to that of the adult. When the adult pattern of convolutions and fissures could be traced in the fetal brain the response was confined to those areas which correspond to the primary and secondary somesthetic areas of the adult sheep. Responses in the cortical associative areas were never obtained. In some older fetuses (85—120 days) the response also displayed a long-latency afterdischarge following the initial primary complex. The finding that the primary response and the afterdischarge showed selective reactions to repetitive stimulation and were accompanied by single unit activity of different characteristics indicates that these two electrocortical activities are generated by different subcortico-cortical mechanisms. On the basis of these findings it has been suggested that the specific and nonspecific afferent inputs to the somatosensory cortex have a

differential rate of maturation.

From a fetal age of around 100 days, the evoked surface response changed from a monopolar negative to a positive negative form similar to that of the adult. The results from the laminar potential analysis lend support to the view that the reappearance of the surface positivity results from an unmasking of a pre-existing excitatory generator located in deep cortical layers. In these later stages of development the cortical neurons were capable of responding with repetitive discharges to tactile stimulation. The observations of the present study suggest that, in the perinatal sheep, the somatosensory cortex has reached a comparatively high degree of functional maturation.

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ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 395

*From the Department of Medical Pharmacology
University of Uppsala, Sweden*

**Monoaminergic Influence on
Testosterone -Activated Copulatory Behavior
in the Castrated Male Rat**

By

Carl Olof Malmnäs

- I Testosterone-activated copulatory behavior in the castrated male rat.
- II. Monoamine precursors and copulatory behavior in the male rat.
- III. Copulatory behavior in the male rat after impaired monoaminergic neurotransmission.
- IV Effects of LSD-25, clonidine and apomorphine on copulatory behavior in the male rat.

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Co-existence or no existence
that is the question : if no copulation
no population

(Partly from Piet Hein)

To my father

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The papers I II III and IV will in the papers be referred to as Malmöa 1973a 1973b 1973c and 1973d respectively

TESTOSTERONE-ACTIVATED COPULATORY BEHAVIOR IN THE CASTRATED MALE RAT

By

CARL OLOF MALMÖKS

INTRODUCTION

The predominant behavior pattern which is displayed by the male rat during heterosexual intercourse is a repetitive mounting of the female partner. On mounting the male clasps the flanks of the female and exerts pelvic thrusts. When mounted by a male the receptive female raises her perineal region which permits the male to achieve intromission of the penis into the vagina. Although this posture is always displayed by the receptive female when mounted, every mount does not result in intromission. When intromission occurs a vigorous backward lunge is shown by the male when he dismounts. Dismounts after intromission are followed by genital licking while this behavior is less regular and of shorter duration when intromission has not been achieved. After a certain number of intromissions (5-15) the ejaculatory reflex is elicited, recognized by a prolonged mount with a vigorous clasp around the female followed by a slow dismount and subsequent extended genital licking. After ejaculation there is a certain time interval (refractory period) until the male starts to mount the female again.

Copulatory behavior in the male rat is dependent on gonadal hormones. After castration there is a gradual decline in copulato-

ry performance which can be restored to the pre-castration level by testosterone treatment (Shapiro 1937; Stone 1939; Beach and Holz-Tucker 1949). The propionic acid ester of testosterone (TP) is most commonly used in this context.

Previously neuropharmacological studies on heterosexual copulatory behavior in the male rat have exclusively been conducted in the intact subject (Zimbaro and Barry 1958; Gillett 1960; Sourlaires 1963; Bignami 1966; Butcher et al. 1969; Leavitt 1969; Dewsbury and Davis 1970; Whalen and Luttge 1970; Ahlertius et al. 1971; Hyyppä et al. 1971; Salis and Dewsbury 1971; Tagliamonte et al. 1971; Dewsbury 1972; Dewsbury et al. 1972). The testing procedure which has been used in these studies usually allows the subject to copulate until the occurrence of one or more ejaculations. Interest has been focused on effects of drugs on parameters of the established copulatory behavior and the post-ejaculation refractory period in as a rule vigorously copulating subjects. However it is an advantage to work with castrated animals supplied with exogenous hormone in neuropharmacological studies on this behavior:

- 1 drug effects on the gonadal hormone production are avoided and
- 2 it is easier to maintain a submaximal response level in order to allow a stimulatory or inhibitory effect of a drug treatment to be seen.

This investigation deals mainly with pre-ejaculation copulatory (= mounting) behavior in the adult castrated male rat. The main aim was to find a schedule of TP treatment that induced and could maintain a submaximal response with respect to the percentage of subjects that displayed mounting behavior.

MATERIAL AND GENERAL METHODS

Subjects

Experimental subjects. About 450 male Wistar rats weighing 350-450 grams when used in experiments (purchased as Specific Pathogen Free Møllegaard Ejby, Denmark). The animals were housed three in each cage (Macrolon[®] 34x40x15 cm) in a room with forced venti-

mance during these 3 min. The observation cages were arranged so that 3 subjects could be tested simultaneously. From the records taken the following measures could be determined:

- 1 Mount percentage
The percentage of subjects which displayed at least one mount with or without intromission during the test; only mounts with evident pelvic thrusts were recorded
- 2 Mount latency^{*}
The time (expressed as hundredths of a min.) from the introduction of the female to the first mount with or without intromission (see fig. 1:B)
- 3 Number of mounts per min.^{*}
Total number of mounts with or without intromission during the test divided by the length in min. of the complete 3 min. test is a total number of mounts/3. Thus subjects which ejaculated before the 3 min. period was ended were excluded from this calculation
- 4 Intromission percentage
The percentage of subjects which displayed intromission within 3 min. from the introduction of the female
- 5 Intromission latency^{*}
The time (expressed as hundredths of a min.) from the introduction of the female to the occurrence of the first intromission if this occurred within 3 min. from the introduction of the female
- 6 Intromission ratio
The percentage of mounts with intromission of the total number of mounts. Only subjects which displayed intromission within 3 min. from the introduction of the female were included
- 7 Ejaculation percentage
The percentage of subjects that displayed ejaculation within 3 min. from the first mount
- 8 Ejaculation latency^{*}
The time from the first intromission to ejaculation (fig. 1:E)

Note: Parameters 2, 3, 5, 6 and 8 were recorded for each individual subject that displayed the behavior in question while parameters 1, 4 and 7 are percentage numbers based on several subjects

Observation cages

Tests were performed in rectangular wooden cages (40x60x40 cm) with a 27 cm high plexiglass front and a wire mesh top. On the Masonite[®] floor there were wooden shavings. Animals were brought into the cage through the 13 cm wide opening between the plexiglass front and the top cover.

^{*}Footnote: Times were measured by means of a stop-watch the scale of which was divided into hundredths of a min.

The Experimental Design

A Chronological schedule of treatment

1 Pre-castration tests: The experimental subjects were given opportunity for sexual experience. They were tested with sexually receptive females for 20 min twice weekly. This regimen was continued until the first ejaculation was observed. 2 Castration: Within a few days after the first recorded ejaculation the subjects were castrated by scrotal incision under ether anesthesia. At the time of castration they were about 100 days of age. 3 Post-castration tests: a) After castration the subjects were tested for copulatory activity once weekly. From this point on the subjects were tested according to the testing procedure stated above. b) When in two successive post-castration tests the mount percentage of the batch was less than 50% the animals were given TP once weekly. The copulatory tests were performed 3 or 4 days after the TP injection.

B Statistical methods

1 Copulatory behavior: A pre-experimental test means the last test preceding a treatment and an experimental test means the test following a treatment. Blank treated subjects were tested parallel to the experimental subjects. The pre-experimental test was used as comparison when a statistical significance of the effect of an experimental treatment was calculated. Thus the subjects served as their own controls. Non-parametric statistical tests were used (Siegal 1956) namely the sign test (to analyze the significance of differences in mount percentage, intromission percentage and ejaculation percentage) and the Wilcoxon matched-pairs signed-ranks test (to analyze the significance of differences in mount latency, number of mounts/min, intromission ratio and the number of mounts each min in exp A:2). 2 For the analysis of differences in motility between experimental and control subjects the Mann-Whitney U test was used.

The 5 per cent level of significance was used (two-tailed probabilities).

C Pre-experimental states of subjects

- I Sexually experienced intact subjects
- IIa Castrated subjects (never given TP) drawn from a batch of animals with a mount percentage less than 50%
- IIb Castrated subjects which had been subjected to a submaximal TP treatment but not given TP during the last 3 weeks prior to the experiment
- III Castrated subjects which were tested once weekly and maintained on submaximal TP treatment given 3 or 4 days prior to each weekly test

Since there was no significant difference in response between subjects in pre-experimental I = IIa and IIb in exp C1 and C3

the data were pooled

Injected Materials

The hormones used were testosterone propionate (TP) free testosterone estradiol benzoate and progesterone (Organon) dissolved in olive oil free dexamethasone (Organon) suspended in olive oil and thoroughly shaken before injection and porcine ACTH [short acting (amorphous) Acton^R Ferring and long acting (zinc chloride prolonged release) Cortrophin^R prol Organon through Pharmacia] diluted in 0.9% saline. All injections were given subcutaneously in a volume of 1 ml/kg body weight.

STUDIES ON THE EXPERIMENTAL PROCEDURE

A. COPULATORY BEHAVIOR IN THE INTACT MALE RAT

Since the main interest in this investigation was focused on pre-ejaculation copulatory behavior the design of the testing procedure was made with regard to this aim. The duration of a test should be long enough to allow mount latency and the number of mounts/min to be determined. The length of time a test should continue in order to meet these requirements was tested in exp. A. (See also exp. D4 and D5 for TP treated castrated males.)

Experiment A 1 Distribution of Mount and Ejaculation Latencies

Procedure

30 subjects in pre-experimental state I (see Methods) were allowed to copulate until they achieved ejaculation.

Results

The distribution of mount latencies is given in fig. 2. The majority of subjects mounted the female within half a min. after the female was introduced (median 10 min./100) and all subjects had a

Fig 2 Mount latencies Distribution in sexually experienced intact male rats (N = 30)

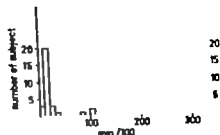


Fig 3 Ejaculation latencies Distribution in sexually experienced intact male rats (N = 30)

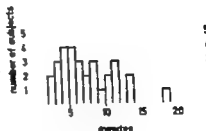


Fig 4 The number of mounts each min (mean \pm s.e.m.) during the first 10 min counted from the first mount. Sexually experienced intact male rats with ejaculation latencies greater than 10 min. (N = 18)



mount latency shorter than 2 min. The distribution of ejaculation latencies is given in fig 3. No subject ejaculated within the first or second min. after the first intromission while during the third min., two subjects ejaculated. The median ejaculation latency was 6.3 min. All subjects ejaculated within 20 min. from the first intromission.

Experiment A 2 The Number of Mounts Each Min during the First 10 Min

Procedure

From a batch of subjects in pre-experimental state I (see Methods)

18 males with an ejaculation latency in an earlier run of between 10 and 20 min. were selected. The number of mounts each min. for 10 consecutive min. counted from the first mount was determined.

Results

The distribution of the average number of mounts each min. during the first 10 min. is given in fig. 4. During the first 4 min. there was a gradual decline in the frequency of mounts from 5.3 the first min. to 3.6 the fourth min. ($p < 0.01$). After 4 min. no further significant decrease was seen.

Conclusion from exp. A 1 and A 2. The data show that 3 min. of testing is enough to cover mount latency. Counted from the first mount, 3 min. is also a suitable time for the number of mounts/min. to be determined since very few subjects ejaculated within this time.

B THE EFFECT OF CASTRATION

Experiment B 1 Exploratory Behavior

Procedure

6 castrated and 6 sham-castrated subjects were used 5 months after surgery. One male at a time was run for 10 min. in an Animex^R activity meter (Svensson and Thiene 1969) (movements of an animal across a tuned oscillator coil system results in a change of the tuning which is recorded as a count). The kind of motor activity displayed during the 10 min. period was mainly exploring of the cage. The experiment was performed during the dark period of the day.

Results

There was no significant difference in the number of counts scored by subjects in the two treatment categories (table I). Thus this experiment shows that the type of motor activity which is measured

TABLE I The effect of castration on motor activity in male rats. Surgery was done 5 months prior to the measurement. One animal at a time was run for 10 min. in an Ardenex activity meter

MOTOR ACTIVITY					
Treatment	Count /10 min	s	m	p	N
Sham-castrated	923	41		0.05	6
Castration	963	64			6

by this method is not decreased 5 months after castration.

Experiment B 2 Copulatory Parameters

Procedure

65 subjects in pre-experimental state I (see Methods) were subjected to a 3 min. standard copulatory test (pre-exp test). One day after this test 52 of the subjects were castrated and 13 were sham-operated. Tests for copulatory behavior were conducted once weekly starting one week after surgery.

Results

The results are given in fig. 5 and table II. All sham-castrated subjects mounted and had intromissions throughout the 7 week experimental period. Compared to the pre-exp test no significant changes of the parameters occurred except for the intromission latency which was significantly reduced. In contrast the castrated subjects showed a continuous reduction in mount percentage and intromission percentage. Five weeks after castration 50% of the castrated males mounted the female. The number of mounts/min. gradually declined and the mount latency and intromission latency became continuously longer after castration. The intromission ratio however remained unchanged. The intromission latency was significantly increased at 1 through 6 weeks after castration but not at 7 weeks. The data obtained at 7 weeks were influenced by a

TABLE II The effect of castration on copulatory behavior in male rats. The pre-experimental test was performed one day before surgery and the experimental test at 7 weeks post-castration. Md = median. a = p 0.05 b = p 0.01 c = p 0.001

Copulatory parameter	Treatment					
	Sham-castration			Castration		
	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>						
Percentage	100	100	13	100	44 ^a	52
Latency ^{Md}	10	10	13	10	40 ^b	23
No/min ^{Md}	3.3	3.3	12	4.0	2.0 ^b	21
<u>Intromission</u>						
Percentage	100	100	13	100	23 ^c	52
Latency ^{Md}	30	15	13	15	40	12
Ratio ^{Md}	63	63	13	60	69	12
<u>Ejaculation</u>						
Percentage	0	8	13	6	8	52

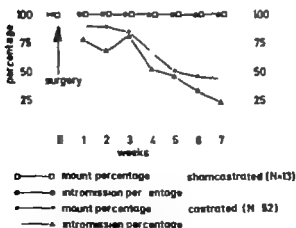


Fig. 5 The effect of castration and sham-castration on mount percentage and intromission percentage in the male rat

relatively great reduction in one subject and by the smaller number of males which displayed intromission

At the beginning of each test all subjects approached the female irrespective of subsequent mounting or non mounting. If they did not mount the female a major part of the first min was devoted to anogenital sniffing of and other social interaction with her. Regarding the exploratory activity oriented towards the environment there was no obvious decrease after castration.

C THE EFFECT OF A SINGLE INJECTION OF TESTOSTERONE PROPIONATE (TP)

The following experiments (C 1-3) were carried out in order to study the effect of different doses of TP on copulatory behavior in the castrated male rat with regard to latency to onset, duration and magnitude of response

Experiment C 1 The Latency to Onset of the Hormone Effect

Procedure

Out of 212 subjects in pre-experimental states IIA and IIB 120 did not mount in the first test (= Day -1). These 120 subjects were then tested once daily for another 4 consecutive days. Six hours before the second test (= Day 0) a single injection of TP free testosterone (T) or oil vehicle was given as shown in fig. 6

Results

Fig. 6 shows the distribution in time of the appearance of the first mount after the different treatments given. The number of animals mounting after TP treatment at 6 and 30 hours after the injection was not significantly different from the oil blank results. A consistent effect in all TP treated groups was that the distribution reached its highest value 54 hours after the TP injection. This peak was not seen in the oil treated controls. A rough estimate of the median latency for TP to activate mounting

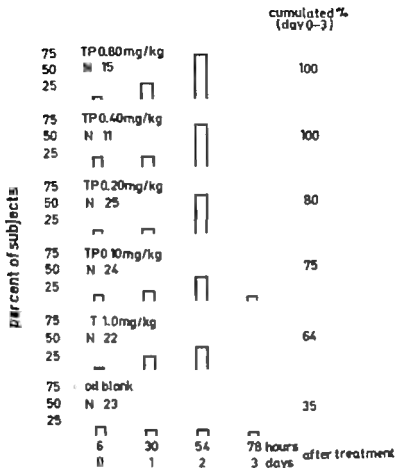


Fig. 6 Latency to onset of the hormone effect in non-mounting subjects. Distribution of the time interval from the administration of TP or T (free testosterone) to the appearance of the first mount. The injections were given 6 hours before the test at Day 0 and the subjects were tested once daily for 4 consecutive days until the appearance of the first mount. Castrated male rats in pre-experimental state IIA and IIB (N = 120).

in the subjects is about 30-54 hours. Essentially the same results were obtained after treatment with free testosterone 1.0 mg/kg.

Experiment C 2 The Duration of the Effect on Mount Percentage of a Single Dose of TP

Procedure

61 subjects in pre-experimental state IIA were used. They were divided into 4 groups which received either TP 0.10 (N = 15) 0.20 (N = 15) or 0.40 (N = 21) mg/kg or oil blank solution (N = 10). The TP treatment was given 6 hours before the test at Day 0. Tests for copulatory behavior were conducted at Day -1 0 1 2 3 5 10 15 and 20.

Results

Mount percentages for the different days after treatment are given in fig. 7. At the day before treatment the response rate was about 40-50 % for the different treatment categories. No effect of the testosterone treatment was seen at Day 0 and 1. An evident in-

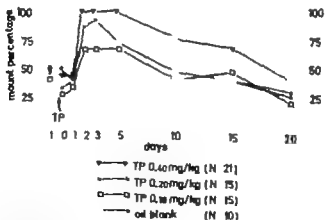


Fig. 7 The duration of the effect of a single dose of TP on mount percentage in castrated male rats (N = 61). The treatments were given 6 hours before the test at Day 0.

crease in response was obtained at Day 2 and this response was maintained in all treatment categories at Day 3. At Day 10 there was still an evident effect after TP @ 40 mg/kg but not after the lower doses.

Experiment # 3 The Effect of Single Doses of TP on Copulatory Parameters

Procedure

126 subjects in pre-experimental states IIA and IIB were used. They were divided into 3 groups. These groups were given either oil vehicle (N = 40), TP @ 10 mg/kg (N = 65) or TP @ 40 mg/kg (N = 21) and were tested for copulatory behavior 3 days after the injections.

Results

Both TP doses used induced a significant increase in mount percentage while no significant effect on this parameter was seen in the oil treated controls (table III). The intromission percentage and the number of mounts/min increased significantly after TP @ 40 mg/kg but no significant effect was obtained after TP @ 10 mg/kg. A significant reduction in mount latency was obtained already after TP @ 10 mg/kg and also after TP @ 40 mg/kg. In the oil treated controls the number of mounts/min. decreased between the pre-experimental and experimental test situation but in other respects there were no significant changes between the two tests.

THE EFFECT OF WEEKLY TP INJECTIONS

In this section (exp # 1-8) different aspects of copulatory behavior were studied using single TP doses given repeatedly once weekly.

TABLE III. The effect of single doses of testosterone propionate (TP) on copulatory behavior in oestrated male rats. TP was given 3 days before the experimental test which was conducted one week after the pre-experimental test. Md = median, s = \bar{p} 0.05 b = \bar{p} 0.01, c = \bar{p} 0.001

Copoly parameters	Treatment mg/kg								
	Oil blank 0.4 ml			TP 0.10			TP 0.40		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>									
Percentage	50	45	40	42	62 ^b	65	43	100 ^c	21
Latency ^{Md}	63	53	14	60	15 ^a	26	40	15	9
No/min ^{Md}	2.7	2.0 ^b	14	2.7	2.3	26	2.0	3.3	9
<u>Intromission</u>									
Percentage	38	38	40	28	38	65	29	90 ^c	21
Latency ^{Md}	53	45	8	70	40	13	38	18	6
Ratio ^{Md}	75	60	8	50	40	13	64	46	6
<u>Ejaculation</u>									
Percentage	0	0	40	0	0	65	0	0	21

Experiment D 1 Day of Maximal Response

Procedure

28 subjects in pre-experimental state III were used. The subjects were divided into 3 groups and were given TP 0.14 mg/kg once weekly for 3 consecutive weeks. At the first injection of the present experiment 2 weeks had elapsed since the last TP injection. Each group was subjected to a different day of testing every week either 1, 3 or 5 days after the TP injection.

Results

The results obtained at the different days of testing after TP injections are given in table IV. Only very small differences in the recorded parameters were found between the various days of testing. The only significant one was a longer mount latency at day 1 than at day 3 and day 5. Although there were very small dif-

TABLE IV The copulatory behavior at Day 1, 3 and 5 after a weekly injection of testosterone propionate (TP) 0.14 mg/kg in castrated male rats. Md = median
 $\alpha = p < 0.05$

Copulatory parameter	Day after TP treatment					
	Day 1 p (Day 1-3)	N	Day 3 p (Day 3-5)	N	Day 5 p (Day 1-5)	N
<u>Mount</u>						
Percentage	54	28	57	28	50	28
Latency ^{Md}	70 ^a	13	30	13	30 ^a	12
Mo/min ^{Md}	2.7	13	2.3	12	2.5	12
<u>Intromission</u>						
Percentage	36	28	36	28	36	28
Latency ^{Md}	47	6	40	6	30	6
Ratio ^{Md}	50	6	72	6	54	6
<u>Ejaculation</u>						
Percentage	0	28	0	28	4	28

forces between the different days of testing it was considered an advantage to have a standard time between the TP injection and the copulatory tests. In all subsequent experiments copulatory tests were performed 3 or 4 days after the TP injection.

Experiment II 2 Dose Response Relationship

Procedure

52 subjects in pre-experimental state IIA were used. They were divided into 4 groups and were subjected to 5 weekly tests 3 or 4 days after TP administration. Four treatment categories were used: oil blank solution, TP 0.050 mg/kg, TP 0.10 mg/kg and TP 0.20 mg/kg. Each group was run at one dose level only.

Results

In the pre-exp. tests (fig. 8 week 0) the mount percentage was

about 20-25 % for all groups. The mount percentage of the group which received TP 20 mg/kg once weekly increased to 80 % and remained on this level from week to week. The response after TP 10 and 0.050 mg/kg stabilized at 40 % and 33 % respectively. The oil treated controls decreased slightly in response level from 20 % to 10 % after 4 weeks. It is evident from this experiment that a weekly maintenance dose of TP 20 mg/kg or less will give a submaximal response for at least 5 weeks. It is also evident that the response (mount percentage) is dose-dependent.

Experiment D 3 The Long Term Effect of TP Given Once a Week

Procedure

24 subjects in pre-experimental state IIA were used. They were given TP 0.14 mg/kg/week for 20 consecutive weeks. Copulatory tests were performed 3 or 4 days after TP injections.

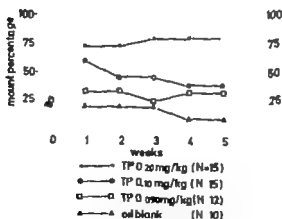


Fig 8 Dose-response relationship of weekly administered doses of TP on mount percentage in castrated male rats (N = 52). Copulatory tests were performed 3-4 days after the weekly TP treatment which started at week 1.

Results

The mount percentage during the entire test period is given in fig 9. The maximal response 67 % was obtained in the first and second week and the lowest response 41 % was obtained at week 6. Throughout the testing period the response was within this range. There was no tendency towards a systematic shift of the response with time.

Experiment D 4. Distribution of the Copulatory Parameters in Subjects Kept on Weekly TP Treatment

Procedure

156 subjects in pre-experimental state III were used and the TP dosage was 0.14 mg/kg/week. The subjects had been maintained on this schedule of TP treatment for 4 months. The testing procedure in this experiment was different in one respect from the one stated in Methods: subjects which had not performed any intromission during the ordinary 3 min. of testing were allowed another 10 min. of heterosexual contact in the observation cage.



Fig. 9. The long term effects on mount percentage of a weekly administered dose of TP. The TP dose used was 0.14 mg/kg/week given 3 or 4 days before the copulatory tests starting at week 1. Castrated male rats ($N = 24$).

Distribution of the Copulatory Parameters in Subjects Kept on Weekly TP Treatment (0.14 mg/kg)

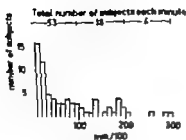


Fig 10 Mount latencies Distribution in TP treated castrated male rats (N = 75)

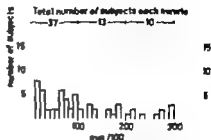


Fig 11 Intromission latencies Distribution in TP treated castrated male rats (N = 60)



Fig 12 Number of mounts/min Distribution in TP treated castrated male rats (N = 75)

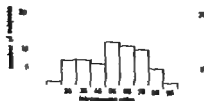


Fig 13 Intromission ratios Distribution in TP treated castrated male rats (N = 60)

Results

A. Ordinary testing procedure: No subject ejaculated during the test. The mount percentage was 48 % and the intromission percentage was 38 %. As shown in fig. 10 and 11 the majority of subjects had mount and intromission latencies shorter than 1 min. (medians 40 and 85 min./100 resp.). The distribution of mounts/min. is given in fig. 12. The median value is 2.7 and there is a fairly symmetrical distribution around this value. The distribution of intromission ratios is given in fig. 13. Also this distribution is fairly symmetrical around a median value of 55.

B. Extended time of testing: Of 81 subjects which did not mount during the ordinary 3 min., only 3 subjects mounted when allowed another 10 min. of testing. Of the 15 subjects which mounted without intromission during the ordinary 3 min., 4 subjects had at least one intromission during the additional 10 min.

Experiment D 5. Distribution of Ejaculation Latencies in Subjects Kept on a Submaximal TP Treatment for Several Months

Procedure

23 subjects in pre-experimental state III were used and the TP dosage was 0.15 mg/kg/week. The subjects had been maintained on this schedule of testing and TP treatment for 8-11 months. Subjects which performed at least 3 intromissions during a standard test (3 min.) were allowed to continue copulation until the occurrence of ejaculation.

Results

The distribution of ejaculation latencies is given in fig. 14. No subject ejaculated within 3 min. from the first achieved intromission. Most subjects had ejaculation latencies shorter than 10 min. with a median of 8.9 min. All subjects ejaculated within 30 min. from the first intromission.

Experiments D & E The Acute Effects of Free Testosterone

Procedure

57 subjects in pre-experimental state III were used. They had been maintained on a TP dosage of 0.15 mg/kg/week for 8 months. This dose of TP was also given 3 days before the experimental test of the present experiment. One half of the subjects was given free testosterone 1.0 mg/kg one hour before the experimental test and the other half had olive oil 1.0 ml/kg given at the same time before test. Four hours later all subjects were retested. Two weeks after these tests the experiment was repeated but at this time the treatments were reversed; i.e. subjects which had received free testosterone at the first test were now given oil and vice versa. In the statistical analysis of this experiment the test values obtained after oil treatment were compared to the test values obtained after treatment with free testosterone.

Results

The results are given in table V. Compared to oil treatment no statistically significant effect was found after free testosterone 1.0 mg/kg neither at 1 nor at 5 hours after its injection. It can thus be concluded that an addition of a comparatively high dose of free testosterone has little or no effect within five hours from its administration on the copulatory parameters recorded.

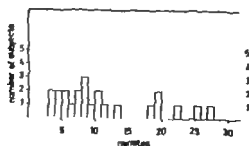


Fig. 14 Ejaculation latency distribution in TP treated castrated male rats (N=23)

TABLE V The effect of free testosterone (T) on copulatory behavior in castrated male rats maintained on a TP treatment of 0.15 mg/kg/week. Copulatory tests at zero hour MD = median.

Copulatory parameter	Treatment mg/kg					
	Free testosterone 1 0			Free testosterone 1 0		
	at -1 hrs			at -3 hrs		
	Oil	T	N	Oil	T	N
<u>Mount</u>						
Percentage	56	53	57	47	46	57
Latency ^{MD}	15	13	25	10	10	17
Mo/min ^{MD}	2 0	2 3	25	2 3	2 3	17
<u>Intromission</u>						
Percentage	39	35	57	23	21	57
Latency ^{MD}	23	20	14	15	20	7
Ratio ^{MD}	60	50	14	57	23	7
<u>Ejaculation</u>						
Percentage	2	2	57	2	2	57

Experiment D 7 The Effect of Increasing the Dose of TP

Procedure

84 subjects in pre-experimental state III were used. They had been maintained on a weekly TP dose of 0.15 mg/kg for 9 months prior to this experiment. In this experiment 44 of the subjects received TP 0.20 mg/kg 3 days before testing instead of the usual dose of 0.15 mg/kg while the other 40 subjects were given the same dose of TP that they had been given previously.

Results

As is shown in table VI there was a significantly increased mount percentage and a decreased mount latency by an increased dose of TP while other parameters were not significantly changed. This experiment shows that the subjects still after several months of a subradical TP treatment have the capacity to display an increased response to an increase of the TP dose.

TABLE VI The effect on copulatory behavior of increasing the dose of testosterone propionate (TP) in castrated male rats maintained on a submaximal response level with respect to mount percentage by means of weekly TP injections. Md = median.
 $\alpha = p < 0.05$

Copulatory parameter	TP treatment mg/kg					
	0.15			0.20		
	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>						
Percentage	43	38	42	48	62 ^a	45
Latency ^{Md}	15	15	13	15	10	17
Mo/min ^{Md}	2.7	3.0	13	2.5	3.0	16
<u>Intromission</u>						
Percentage	31	36	42	29	38	45
Latency ^{Md}	43	43	8	25	25	11
Ratio ^{Md}	59	55	8	57	71	11
<u>Ejaculation</u>						
Percentage	0	0	42	2	0	45

Experiment D 8 The Effect on Mount Percentage of Discontinued TP Treatment

Procedure

52 subjects in pre-experimental state III were used. They had been kept on a weekly TP treatment of 0.14 mg/kg for 8 months prior to this test and were on a stable response level. In this experiment 28 of the subjects were given oil vehicle instead of TP one week and the other 24 subjects had their usual TP treatment (test 2). One week after test 2 all 52 subjects were given TP 0.14 mg/kg (test 3).

Results

The effect of discontinued TP treatment on mount percentage is shown in fig. 15. In subjects which were maintained on a weekly TP treatment the mount percentage was kept at roughly the same

level in test 1 through 3. Discontinued TP treatment (test 2) brought about a significant decrease in mount percentage from 54% to 14% ($p < 0.001$ sign test). The response was restored to almost the same level as in test 1 when TP was given 2 weeks after the last TP treatment (test 3).

E THE INFLUENCE OF THE ADRENALS

Neuropharmacological agents are likely to interfere with the adrenal steroid hormone production. In this section (exp. E 1-3) the extent to which changes of the adrenal function could influence copulatory behavior in the submaximal TP treated castrated male rat was studied.

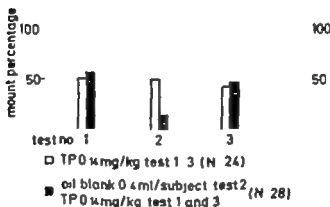


Fig. 15 The effect of discontinued TP treatment on mount percentage in castrated male rats maintained on submaximal response level with respect to this parameter by means of weekly TP injections (0.14 mg/kg). Copulatory tests were performed 3 days after the treatment. Test 1 - 3 consecutive weekly tests.

TABLE VII The effect of adrenalectomy on copulatory behavior in castrated male rats maintained on a subnodal response level with respect to mount percentage by means of weekly testosterone propionate treatment (0.14 mg/kg). Adrenalectomy was performed one day after the pre-experimental test followed one week later by the experimental test. $\bar{M} \pm$ median

Copulatory parameters	Treatment					
	Anesthesia			Adrenalectomy		
	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>						
Percentage	93	53	36	40	37	35
Latency ^{Md}	20	30	15	20	20	8
Mo/min ^{Md}	2.7	2.3	15	2.3	2.7	8

<u>Intrusion</u>						
Percentage	50	44	36	31	31	35
Latency ^{Md}	65	75	10	160	60	7
Ratio ^{Md}	74	62	10	43	40	7

<u>Ejaculation</u>						
Percentage	0	0	36	0	0	35

Experiment E 1: The Copulatory Response One Week After Adrenalectomy

Procedure

71 subjects in pre-experimental state III were used. They had been maintained on a TP dose of 0.14 mg/kg/week since 4 months. One day after the usual weekly test 35 subjects were adrenalectomized under ether anesthesia and 36 subjects were subjected just to the ether anesthesia. One week after these treatments the animals were tested in the regular testing schedule after TP 0.14 mg/kg. The adrenalectomized subjects were provided with 0.9% saline and tap water in their home cages.

Results

The results are given in table VII. One week after adrenalectomy the subjects showed a reduction in the general vigour: They moved slowly, had a tangled fur and had lost about 5% of their body weight. Despite the decrease in general vigour, no change was seen in the copulatory parameters at that time.

Experiment E 2: The Effect of Long Acting ACTH and Dexamethasone

Procedure

- 108 subjects in pre-experimental state III were used. They had been maintained on a TP dose of 0.14 mg/kg/week for 3 months. Three treatment categories were used (N = 36 for each category):
1. ACTH 20 IU/kg (third international standard, slow release zinc chloride preparation)
 2. free dexamethasone 2.0 mg/kg (oil suspension)
 3. saline 0.4 ml/animal

The treatments were given 3 days before the copulatory tests.

Results

The results are given in table VIII. The saline treated subjects remained on their pre-experimental response level while significant changes occurred in both ACTH and dexamethasone treated subjects. After ACTH treatment there was a significant increase in mount percentage, intromission percentage and the number of mounts/min. The latencies to the first mount and first intromission were significantly reduced. In contrast, subjects treated with dexamethasone did not display any change in mount percentage or intromission percentage, but there was an increase in the number of mounts/min and an almost significant reduction in mount latency ($p = 0.05$). At the day of testing, the dexamethasone treated subjects had lost about 10% of their pre-injection body weight. They were restless but had in fact a decreased motor activity score compared to controls when tested for 10 min in the Animax^R activity meter (saline = 819 ± 34 , dexamethasone = 687 ± 33 , mean \pm s.e.m., $N = 9$, $p < 0.02$).

TABLE VIII The effect of "long-acting" ACTH and free dexamethasone on copulatory behavior in castrated male rats maintained on a subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment (0.11 mg/kg). The treatments were given 3 days before the experimental test. Md = median. a p < 0.05 b = p = 0.01

Copulatory parameter	Treatment								
	Saline			Dexamethasone			ACTH		
	0.4 ml			2.0 mg/kg			20 IU/kg		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>									
Percentage	47	58	38	58	56	36	42	64 ^a	36
Latency ^{Md}	20	35	13	25	13	18	40	20 ^b	14
Mo/min ^{Md}	3.3	2.3	13	2.3	3.7 ^b	17	2.5	2.9 ^a	14
<u>Intromission</u>									
Percentage	49	42	36	44	53	36	31	58 ^a	36
Latency ^{Md}	80	60	11	60	50	11	140	30 ^b	9
Ratio ^{Md}	57	67	11	59	59	11	47	71	9
<u>Ejaculation</u>									
Percentage	0	0	36	0	3	36	0	3	36

Experiment E 3 The Effect of Short Acting ACTH in Non Adrenalectomized and Adrenalectomized Subjects

Procedure
 30 non-adrenalectomized and 30 adrenalectomized subjects in pre-experimental state III were used. They had been maintained on a TP dose of 0.15 mg/kg for 3 and 10 months respectively. Adrenalectomy was performed one month prior to this experiment. The adrenalectomized subjects were given free dexamethasone 0.2 mg/subject/week and were provided with 0.9% saline and tap water in their home cages.

One half of the subjects in each category was given short-acting" (amphous) ACTH 10 + 5 + 5 IU/kg (third international standard) at 6, 4 and 2 hours before the test respectively while the other half of the subjects was given saline at the same time intervals. Three days after this test the subjects were retested.

Two weeks later the experiment was repeated but this time the treatments were reversed; i.e. subjects which had received ACTH in the first test were now given saline and vice versa. In the statistical analysis of this experiment the test values after saline treatment were compared to the ones after ACTH treatment.

Results

The results are given in table IX. In non-adrenalectomized subjects the number of mounts/min. was significantly increased within 6 hours from the administration of ACTH while the other parameters were unchanged. Three days later however mount percentage was significantly increased and mount latency significantly decreased while no other parameter was significantly changed. In adrenalectomized subjects none of these behavioral changes was observed.

F STUDIES INCLUDING OTHER BEHAVIOR PATTERNS THAN COPULATORY

This section (exp. F 1) is devoted to the normal behavior displayed during copulatory tests by castrated subjects kept on a submaximal TP treatment.

Experiment F 1 A Simple Way to Score the Orientation of Activity during Copulatory Tests

Procedure

179 subjects in pre-experimental state III were used. They were maintained on a TP treatment of 11.10 mg/kg/week. The records were taken parallel to the records of copulatory behavior. The scores for the following mutually exclusive orientations of activity were recorded:

1 Activity oriented towards the female

This includes anogenital and non-genital sniffing and interaction, pursuit, climbing and mounting with or without intro-

mission

2 Activity oriented towards the environment

This includes exploration of the cage rearing and other kinds of locomotor activity

3 Activity oriented towards the subject itself

This includes genital and non-genital grooming

4 Immobility

Standing still or lying down.

Scoring technique

The different orientations of activity were scored each min during the first 3 min. after the introduction of the female. Each subject was given a score of 1 for each of the 3 min. in which a certain orientation of activity was displayed. The criterion for getting a score was that a certain behavior was displayed for at least 0.05 min consecutively during that min. (Exception: if a subject mounted the female it was given the score of 1 for activity oriented towards the female for that min. even if pursuit plus mount took less time than 0.05 min.) During the 3 min period a subject could thus score a minimum of 0 and a maximum of 3 for each of the different orientations of activity recorded.

Subjects which performed at least one mount ("mounters") and subjects which did not mount ("non-mounters") were placed in separate categories in the analysis of the behavioral data. (Subjects which ejaculated before the end of the 3 min. period were excluded.)

Results

The percentage of subjects which obtained a certain score for orientation of activity is given in fig. 16. The score profiles were different for the two categories of subjects. At the beginning of each test all subjects approached the female irrespective of subsequent mounting or non-mounting. If they did not mount the female a major part of the first minute was devoted to anogenital sniffing of and other social interaction with her. Among "mounters" the most frequent score for orientation towards the female was 3.

while 1 was the most frequent score among "non-mounters". Since all subjects displayed orientation towards the female during the first minute after her introduction this means that most "mounters" also paid attention to her during the next 2 min while most "non-mounters" did not display this behavior during any of the next 2 min. The average score for environment-oriented activity was greater among "non-mounters" although the most frequent score was 2 for both categories of subjects. For activity oriented towards the subject itself "mounters" mainly scored 3 while non-mounters mainly scored 1 or 2. The criterion for getting a score for immobility was fulfilled by only one out of the 179 subjects. This subject happened to be a "non-mounters".

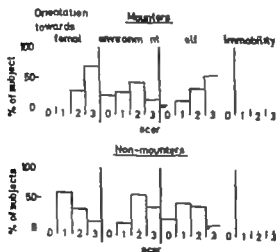


Fig 16 Orientation of activity during copulatory tests in castrated male rats maintained on a submaximal response level with respect to mount percentage by means of weekly TP treatment (0.10 mg/kg). Comparison is made between mounters and non-mounters ($N = 73$ and $N = 106$). A subject was given the score of 1 for each of the 3 min in which a certain behavior was displayed for the last 0.05 min. For further details see text.

DISCUSSION

The main aim of the present investigation was to find a schedule of TP treatment that induced and could maintain a submaximal response with respect to the percentage of subjects that displayed pre-ejaculation copulatory behavior. The different steps of the investigation will be discussed separately.

The Testing Procedure

A time schedule with an upper limit of 3 min. before the first mount and 3 min. of copulation allowed after the first mount was used in the copulatory tests. This testing procedure seems to be appropriate for TP treated castrates because:

1. Within the first 2 min. all but a very small number of the mounting subjects had performed their first mount (fig. 10) i.e. mount latencies were shorter than 3 min.
 2. Counted from the first mount 3 min. was also a suitable time for the number of mounts/min. to be determined since: A the time was sufficient for several mounts to be performed (fig. 12) and B few or no subjects ejaculated within this time (fig. 14). Furthermore this short time of testing should be appropriate for pharmacological studies since:
 3. The behavioral effects of even short acting pharmacological agents will change little during the course of a test.
- A practical consequence of this testing procedure is that:
4. Several subjects can be tested within a restricted time period (30-40/hour).

However one might assume that 3 min. of heterosexual contact/week is such a slight reward that even an intact subject would stop copulating within a few weeks under this regimen. No support for such an assumption was obtained from the present experiments. Intact subjects copulated vigorously for at least 7 weeks under

this regimen (exp B2) and in fact continued to do so for more than a year (unpublished observations)

The Effect of Castration

At 8 months after castration motor activity as measured by the Animax^R activity meter was within a normal range. In contrast within 7 weeks from castration copulatory activity had gradually decreased: there was a reduction in mount and intromission percentages, prolonged mount and intromission latencies, as well as a decreased number of mounts/min. The intromission ratio however was not significantly changed, indicating an adequate ability of penile erection and vaginal intromission. The decreased copulatory scores after castration were not accompanied by a lack of exploring of the female at the beginning of each copulatory test.

The Effect of Single TP Doses

Regardless of the TP dose given to non-mounting subjects, it took several hours (30-54 hours) until mounting behavior was displayed (exp C1). The duration of the TP effect (mount percentage) was dependent on the dose given. Calculated from the day the TP injection was given, the duration of the effect was more than 5 days but less than 10 days at a submaximal dose level, while the duration was longer after a supramaximal dose (exp C2). The amount of TP needed for a significant change to occur was not the same for all copulatory parameters. Exp C3 (and D7) shows that mount percentage and mount latency were significantly changed by a smaller dose of TP than was necessary to change the other parameters significantly.

Weekly TP Injections

The choice of doses and time interval for repeated administration of TP was guided by experience obtained from the experiments with single TP injections. From the results of these experiments it seemed likely that a TP dose of ≈ 20 mg/kg or less given repeatedly once weekly should have little or no cumulative effect with respect to mount percentage. By giving weekly TP injections it was found possible to maintain a submaximal response level for several months without tendencies towards a systematic shift of the response with time (exp D3). Furthermore the level of response was dose-dependent (exp D2).

One may ask if subjects kept for long time periods on a submaximal response level remain sensitive to a change of the TP supply or if the conditioning to the regular testing procedure is so strong that a temporary change of the TP supply has little effect upon the response. Exp D7 shows that still after several months of a submaximal TP treatment the copulatory response could be increased by raising the dose of TP and from exp D8 it is clear that a temporary withdrawal of the TP treatment reduced the response. Thus still after several months from the beginning of treatment the subjects were sensitive to changes in the hormonal supply. An injection of free testosterone (1 mg/kg) had no significant effect on the copulatory behavior within 5 hours from its administration. Thus it takes more than 5 hours for testosterone to affect copulatory behavior in subjects kept on a submaximal response level with respect to mount percentage by means of weekly TP treatment.

The Influence of the Adrenals

No significant effect on the copulatory behavior was detected one week after adrenalectomy. This is in accordance with earlier findings of Bloch and Davidson (1968) although their experimental ap-

proach was different. Suppression of adrenal activity by means of a high dose of dexamethasone did not impair copulatory performance either. On the contrary, there was a significant increase in the number of mounts/min and an almost significant reduction of mount latency. Since facilitatory effects of this kind were not obtained by adrenalectomy, these facilitatory effects might be due to direct central nervous effects of dexamethasone itself rather than secondary to adrenal suppression. It can be concluded that if not stimulated more than ordinarily (by drugs or other factors) the adrenals seem not to produce a sufficient amount of steroid hormones to exert a major influence on copulatory behavior in the castrated male rat treated with small doses of TP. On the other hand, ACTH treatment brought about facilitatory effects in several respects. Since a facilitation of copulatory behavior was not seen in ACTH treated adrenalectomized subjects, it is likely that the facilitatory effects on copulatory behavior of systemically administered ACTH were due to an increased adrenal steroid synthesis and release. Notably, within 6 hours from the administration of ACTH the number of mounts/min was significantly increased. The relationship between this effect and an endogenous release of testosterone could be questioned since free testosterone did not have this effect within a similar length of time. Three days after the injection of "short-acting" ACTH the number of mounts/min was not different from control levels but instead mount percentage was increased and mount latency decreased; such effects were also seen after a small increase of the dose of TP (exp. D7). These ACTH experiments suggest that an intense stimulation of the CNS-pituitary-adrenal axis might influence copulatory behavior. It is of course important to be aware of this when effects of drugs on copulatory behavior are considered, especially if they are given several hours before the copulatory test.

Other Behavior Patterns than Copulatory

Drugs that affect copulatory behavior in the male rat might do so either by a primary action on the physiological mechanisms which regulate this behavior and/or indirectly by an influence on e.g. well-being, motor capability or wakefulness. Since little attention has been paid to this matter previously, there is an apparent demand for simple methods to differentiate specific from non-specific effects of neuropharmacological agents on copulatory behavior. The scoring technique introduced in exp. F1 was developed for this purpose. Regarding the orientation of activity displayed by the male rat during copulatory tests, two points in particular should receive careful attention:

1. Immediately after the introduction of the female into the observation cage, all males approached, explored and interacted with her.
2. Very few subjects fulfilled the criterion for "immobility".

If a drug abolishes the initial exploring of the female or if many subjects fulfill the criterion for "immobility", the possibility of non-specific drug-actions on copulatory behavior should be considered.

CONCLUDING REMARKS

The main objective of this investigation was to develop a method appropriate for pharmacological studies on the hormone-activated heterosexual pre-ejaculation copulatory (mounting) behavior in the castrated male rat. The interest was focused on the percentage of subjects which displayed mounting (mount percentage). By means of weekly injections of small amounts of testosterone propionate, mount percentage could be maintained on a stable, submaximal level for several months, the response remaining sensitive to changes in the hormonal supply throughout. The present method seems to meet the requirements for its intended purpose. Since castrates

are used drug effects on the gonadal hormone production are avoided; experimental evidence is presented however that the possibility of drug-interactions with the adrenal function should also receive attention

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MONOAMINE PRECURSORS AND COPULATORY BEHAVIOR IN THE MALE RAT

By

CARL OLOF MALMÖKS

ABSTRACT

Pargyline 40 mg/kg and nialamide 230 mg/kg suppressed testosterone-activated heterosexual copulatory behavior in castrated male rats. DL-5-HTP 1.0 and 2.5 mg/kg inhibited copulatory behavior when combined with a subeffective dose of pargyline (20 mg/kg) whereas L-DOPA 1.0 and 10 mg/kg did not. DL-5-HTP 2.5 mg/kg inhibited copulatory behavior also when given after a combined pretreatment with pargyline (20 mg/kg) and the extracerebral D-carboxylase inhibitor MK486 50 mg/kg. In contrast L-DOPA 2.5 mg/kg facilitated several components of copulatory behavior when given after the same pargyline and MK486 pretreatment while DL-DOPS 3.0, 10 or 30 mg/kg did not facilitate the behavior. It is suggested that testosterone-activated heterosexual copulatory behavior in the castrated male rat is inhibited by functionally increased central nervous serotonin content and facilitated by increased dopamine content.

INTRODUCTION

Heterosexual copulatory behavior in the male rat is dependent on gonadal hormones. After castration there is a gradual decline of copulatory performance which can be restored by testosterone propionate (TP) (Shapiro 1937; Stone 1939; Beach and Bolz-Tucker 1949; Malmöks 1973a).

Recent research has demonstrated effects on certain components of copulatory behavior in intact male rats of neuropharmacological agents affecting synthesis (Tagliamonte et al. 1971; Salis

and Dewsbury 1971; Ahlenius et al 1971) storage (Soulairac 1963; Butcher et al 1969; Dewsbury and Davis 1970; Dewsbury 1972) release (Soulairac 1963; Butcher et al 1969) and breakdown (Tagliamonte et al 1971; Dewsbury et al 1972) of monoamines. These effects might either be due to an action of the drugs on the nervous mechanisms primarily involved in the control of this behavior and/or to other factors such as influence on e.g. locomotor activity or interference with the endogenous steroid hormone production.

TP treated castrated male rats were used in the present investigation. The subjects were given monoamine oxidase inhibitors. These were given either alone or in combination with monoamine precursor amino acids with or without an extracerebral decarboxylase inhibitor. The aim was to study the effects on copulatory behavior of general and selective increases of the monoamine levels and to evaluate the specificity of the effects obtained.

MATERIAL AND METHODS

A procedure has been developed to maintain castrated male rats on a submaximal response level with respect to the percentage of subjects that displayed copulatory behavior. For detailed description see Malmöls (1973a).

Experimental subjects

Experimental animals were about 360 male Wistar rats (350-450 g) when used in experiments, purchased as Specific Pathogen Free Møllegaard Ejby (Denmark), castrated as adults and with proven sexual capacity (at least one ejaculation) before castration. Spayed Sprague-Dawley female rats were used as stimulus objects. They were brought into behavioral estrus by 25 µg estradiol benzoate followed 48 hrs later by 1 mg progesterone a.c. which was given 4-7 h.s. before the copulatory tests. The animals were housed under forced ventilation at 21 ± 1°C and a reversed day-night light cycle (light from 10 pm to 11 am). Commercial rat pellets (Anticimex 210 Sollentuna, Sweden) and tap water were supplied ad lib.

Injected materials

The hormones used were testosterone propionate (TP) estradiol benzoate and progesterone (Organon). The neuropharmacological compounds used were pargyline HCl (Eutonyl Abbott Lab) mianserin HCl (Miansid[®] Pfizer) L- α -(3,4-dihydroxybenzyl)- α -hydrazino propionic acid (MK486 Merck Sharp & Dohme) DL-5-hydroxytryptophane (DL-5-HTP Nutritional Biochemicals Corp) L-3,4-dihydroxyphenylalanine methyl ester HCl (L-DOPA, Hissle) and DL-threo-3,4-dihydroxyphenylserine (DL-DOPS Hissle). The hormones were dissolved in olive oil and the neuropharmacological agents in saline. MK486 and DL-DOPS were dissolved in hot saline to which a few drops of 2 N HCl had been added and the pH was adjusted to 3 and 5 resp. by addition of 2 N NaOH. All injections of hormones were given subcutaneously while the neuropharmacological agents and blank solutions were given intraperitoneally. All in a volume of 1 ml/kg. Doses mentioned in the text and tables always refer to the forms of the compounds stated above.

Testing procedure

Copulatory tests were performed during the dark period of the light cycle (between 2 pm and 5 pm) under dimlight conditions. The male was transferred from the home cage to an observation cage (40 x 60 x 40 cm) and stimulus female was brought in 5 min later. The time allowed from the introduction of the female to the first mount was restricted to 3 min. If the male mounted the female within 3 min the test continued for another 3 min counted from the first mount until ejaculation occurred whichever came first.

Copulatory parameters recorded

Copulatory behavior in the male rat consists of a series of mounts with or without penile intromission into the female vagina terminated by ejaculation. The following measures were taken:

1. Mount percentage
The percentage of subjects that displayed at least one mount with or without intromission; only mounts with evident pelvic thrusts were recorded.
2. Mount latency
The time (expressed as hundredths of a min) from the introduction of the female to the first mount with or without intromission.
3. Number of mounts per min
Total number of mounts with or without intromission during the test divided by 3. Males which ejaculated within 3 min from the first mount were excluded from this calculation.
4. Intromission percentage
The percentage of subjects that displayed intromission within 3 min from the introduction of the female.

5 Intromission latency

The time (expressed as hundredths of a min) from the introduction of the female to the first mount with intromission.

6 Intromission ratio

The percentage of mounts with intromission of the total number of mounts with or without intromission

7 Ejaculation percentage

The percentage of subjects that displayed ejaculation within 3 min from the first mount

8 Ejaculation latency

The time from the first intromission to ejaculation

Note: Parameters 2 3 5 6 and 8 were recorded for each individual subject that displayed the behavior in question while parameters 1 4 and 7 are percentage numbers based on several subjects

Experimental design of copulatory tests

Tests were carried out regularly once weekly. Intact males display mounting in every test under this regimen (Malmöls 1973a) however after castration there is a decline of the percentage of subjects which display mounting (Malmöls 1973a). When in two successive post castration tests the mount percentage of the batch was less than 50% the animals were given TP once weekly. The TP dose was adjusted to induce a stable submaximal response with respect to mount percentage (0.10 - 0.20 mg/kg 3 - 4 days before the copulatory test). In the neuropharmacological experiments the subjects were tested in groups of 9 - 12 members. A group could appear in more than one treatment category but the same treatment was only given once to the same group. At least one drug free test was conducted between two neuropharmacological treatments. Blank treated subjects were always tested parallel to drug treated subjects as a control of the stability of the hormone-induced response. When calculating statistical significance of a treatment-induced change of the behavior in the experimental test comparison was made with the last no-drug test - the "pre-experimental test" - i.e. each subject served as its own control.

Methods to evaluate the specificity of drug induced changes of copulatory behavior

A Motor activity measurements. The expression of copulatory behavior in the male rat is dependent upon intact motor capabilities. Motor activity measurements were made in order to find possible correlations between the effect of a drug treatment on copulatory behavior and motor activity. One male at a time was run for 10 min in an Animex[®] activity meter (Svensson and Thiene 1969) (movements of an object across a tuned oscillator coil system results in a change of the tuning which is recorded as a

count) The kind of motor activity displayed during the 10 min period is mainly of the exploratory type. Motor activity recordings were performed during the dark period of the day in subjects receiving the same TP and drug treatment as subjects in copulatory tests.

8. Scoring of the orientation of activity during copulatory tests. The scores for the following activities were recorded parallel to the records of copulatory behavior:

1. Activity oriented towards the female
This includes anogenital and non-genital sniffing and interaction, pursuit, climbing and mounting.
2. Activity oriented towards the environment
This includes exploration of the cage, rearing and other kinds of locomotor activity.
3. Activity oriented towards the subject itself
This includes genital and non-genital grooming.
4. Immobility
Standing still or lying down.

The occurrence of any behavior which could be assigned to one of these categories was scored during each of the first 3 min. from the introduction of the female. The criterion for getting a score was that a certain behavior was displayed for at least 0.05 min consecutively during that min. (Exception: if a subject mounted the female it was given the score of 1 for female-oriented activity for that min. even if pursuit + mount took less time than 0.05 min.) More than one orientation of activity could be scored each min. During the 3 min. period a subject could thus score a minimum of 0 and a maximum of 3 for each of the different orientations of activity. In the analysis of the behavioral data the subjects were divided into two categories: 1. mounters = subjects which performed at least one mount both in the pre-experimental and experimental test and 2. non-mounters = subjects which neither mounted in the pre-experimental nor in the experimental test. Subjects which ejaculated before the end of the 3 min. period were excluded from this calculation.

statistical analysis

For the analysis of the significance of a treatment-induced change in the experimental test compared to the pre-experimental test non-parametric statistical tests were used (Siegal 1956) namely the sign test (mount percentage, intromission percentage, ejaculation percentage and orientation of activity) and the Wilcoxon matched-pairs signed-ranks test (mount latency, mounts/min, intromission latency and intromission ratio). Data which appear in tables on mount and intromission latencies, mounts/min and intromission ratio are based on subjects which mounted and had intromissions respectively - both in the pre-experimental and experimental test. The Mann-Whitney U test was used for comparison between

- 5 Intromission latency
The time (expressed as hundredths of a min.) from the introduction of the female to the first mount with intromission.
- 6 Intromission ratio
The percentage of mounts with intromission of the total number of mounts with or without intromission
- 7 Ejaculation percentage
The percentage of subjects that displayed ejaculation within 3 min from the first mount
- 8 Ejaculation latency
The time from the first intromission to ejaculation

Notes: Parameters 2 3 5 6 and 8 were recorded for each individual subject that displayed the behavior in question while parameters 1 4 and 7 are percentage numbers based on several subjects

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TABLE 1. The effect of serotonin oxidase inhibitors on copulatory behavior in castrated male rats maintained on subnormal response level with respect to mount percentages by means of weekly testosterone propionate treatment. The drug and saline injections were given 6 hours before the copulatory tests. $MD = median$. $P < 0.001$

Copulatory parameter	Treatment mg/kg					
	Saline 0.4 ml	Pargyline 20	P. rydline 40	Nialamide 125	Nialamide 250	
	Pre-exp Exp N	Pre-exp Exp N	Pre-exp Exp N	Pre-exp Exp N	Pre-exp Exp N	
<u>Mount</u>						
Percentage	82 88 17	78 81 16	78 6 18	78 89 18	83 0 18	
Latency MD	20 15 13	15 20 13	15 48 1	20 35 13	15 (15)	
Mo/mln MD	30 38 13	33 23 13	20 0 3 1	30 23 13	33 (15)	
<u>Intromission</u>						
Percentage	71 88 17	72 78 16	61 0 18	88 56 18	61 0 18	
Latency MD	38 38 6	28 23 10	45 (11)	70 40 7	88 (11)	
Ratio MD	75 50 6	73 50 10	60 (11)	70 70 7	55 (11)	
<u>Regulation</u>						
Percentage	0 0 17	0 0 18	0 0 18	0 0 18	0 0 18	

TABLE II. The effect of noradrenaline precursor (after pargyline pretreatment) on copulatory behavior in castrated male rats maintained on a stimulant response level with respect to mount percentages by means of weekly testosterone propionate treatment. The drug and saline injections were given at 1.5 hours (pargyline) and 0.5 hours (testosterone) before the copulatory tests. M: median.

* $p < 0.05$, $b < p < 0.01$, $c < p < 0.001$

Treatment, mg/kg		Pargyline, mg									
Copulatory parameter	Saline 2x0.4 ml Pre-exp Exp M	Saline 0.4 ml		L-DOPA, 1.0		+ L-DOPA, 1.0		+ DL-5-HTP 1.0		DL-5-HTP 2.5	
		Pre-exp	Exp M	Pre-exp	Exp M	Pre-exp	Exp M	Pre-exp	Exp M	Pre-exp	Exp M
		Exp	M	Exp	M	Exp	M	Exp	M	Exp	M
<u>Mount</u>											
Percentage	72 73 36	72 73 36	78 75 36	75 75 36	81 56 ^a 36	83 67 ^c 36					
Latency ¹ M	30 38 20	35 30 21	30 20 25	50 20 ^b 26	15 20 20	20 40 15					
Re/mile ² M	3.3 4.0 1.8	3.3 3.0 2.1	3.7 3.0 2.5	3.0 3.0 2.6	3.0 1.8 ^a 2.0	3.3 2.3 ^b 1.5					
<u>Intromission</u>											
Percentage	50 50 36	56 64 36	72 64 36	47 67 36	78 46 ^b 36	64 36 ^a 36					
Latency ¹ M	50 35 1.5	30 65 1.3	75 30 21	70 35 14	75 53 14	40 40 11					
Re/mile ² M	62 53 1.5	40 62 1.3	50 60 21	39 57 ^b 14	51 59 14	50 35 11					
<u>Erection</u>											
Percentage	0 5 36	0 0 36	0 0 36	0 0 36	0 0 36	0 0 36					

TABLE III. The effect of succinylate precursors (after pargyline and 1000g pretreatment) on expulsive behavior in ovariectomized rats administered an subcutaneous response level with respect to acute percentage by means of weekly testosterone propionate treatment. The drug and saline injections were given at 1.5 hours (pargyline) 1.0 hours (1000g) and 0.5 hours (precursors) before the expulsive tests (M) medium. $p < 0.05$, $b < p < 0.01$, $c < p < 0.001$

Copolatory parameter	Treatment mg/kg									
	Pargyline 20 mg/kg					Pargyline 20 mg/kg + 1000g 0.4 ml				
	Saline 3 x 0.4 ml	Exp	Pre-exp	Exp	Pre-exp	Exp	Pre-exp	Exp	Pre-exp	Exp
<u>1000g</u>										
Percentage	30	43	10	66	63	33	56	95	39	68
Latency ^a	20	15	18	15	15	19	15	10 ^b	22	13
Ratio ^a	3	7	2	3	2	0	3	0	16	3
<u>1000g + 1000g</u>										
Percentage	37	47	18	60	31	25	61	67 ^b	39	55
Latency ^a	35	33	13	33	43	10	30	13 ^b	15	20
Ratio ^a	50	44	13	34	39	10	60	75	13	42
<u>1000g + 1000g + 1000g</u>										
Percentage	0	0	18	0	0	35	0	18 ^b	39	3

TABLE IV The effect of DL-DOPA (after pargyline and M&M66 pretreatment) on copulatory behavior in castrated male rats maintained on subnormal response level with respect to sexual percentage by means of weekly testosterone propionate treatment. The drug and saline injections were given at 1.5 hours (pargyline), 1.0 hours (M&M66) and 0.5 hours (DL-DOPA) before the copulatory tests M&M66 medium. p 0.05

Treatment mg/kg												
Copulatory parameter	P argyline 20 M&M66 50											
	Saline 3 x 0.4 ml	DL-DOPA 30		DL-DOPA 10		DL-DOPA 30						
	Pre-exp	Exp	M	Pre-exp	Exp	M	Pre-exp	Exp	M	Pre-exp	Exp	M
<u>Mount</u>												
Percentage	55	52	42	37	41	27	47	47	36	53	57	30
Latency ^{Md}	13	15	17	20	10	7	13	10	14	13	40 ^A	12
No/min ^{Md}	2.3	2.0	1.7	2.3	1.7	7	2.8	2.0	1.3	2.3	2.7 ^A	2.2
<u>Intromission</u>												
Percentage	38	36	43	22	30	27	33	44	36	37	37	30
Latency ^{Md}	85	60	9	50	110	4	75	28	8	80	80	6
Ratio ^{Md}	40	36	9	62	40	4	64	58	8	51	45	6
<u>Ejaculation</u>												
Percentage	2	0	42	0	0	27	0	3	36	0	0	30

cent effect on the copulatory parameters recorded (table II)

L-DOPA 10 mg/kg did not significantly change any of the copulatory parameters. However after L-DOPA, 10 mg/kg there was a decreased mount latency and an increased intromission ratio. In contrast DL-5-HTP 1.0 or 2.5 mg/kg significantly decreased mount percentage, intromission percentage and the number of mounts/min. No other parameter was significantly changed.

The higher dose of L-DOPA, 10 mg/kg was the only treatment that evidently affected the overt behavior. With this treatment the subjects had a reduced locomotor activity (see below) and their furs showed piloerection and were slightly wet.

The Effect of Monoamine Precursor Amino Acids after Extracerebral Decarboxylase Inhibition

Due to its poor passage through the blood-brain barrier the decarboxylase inhibitor MK486 more effectively hinders the peripheral increase in monoamine formation after precursor treatment than the central nervous utilization of the precursor (Porter et al. 1962; Bartholini and Pletscher 1969; Henning and Rubenson 1971). MK486 was used to investigate the significance of increased central nervous versus peripheral monoamine levels for the copulatory behavior. The experiments were designed analogously to the pargyline + precursor experiments but MK486 was given as well.

Pargyline 20 mg/kg given 1.5 hours before test followed by MK486 50 mg/kg 1.0 hours before test had no significant effect when combined with saline given 0.5 hours before test (table III). When L-DOPA, 2.5 mg/kg was added to this pargyline + MK486 pretreatment mount percentage and intromission percentage increased significantly. Furthermore mount latency and intromission latency were reduced and intromission ratio was increased. Despite the fact that there was no significant increase of the number of mounts/min. 7 subjects achieved ejaculation (median ejaculation latency 2.1 min) whereas no one ejaculated in the pre-experimen-

tal test. The subjects that achieved ejaculation did not have more intromissions in the experimental test than they had in the pre-exp test. Quite different effects were obtained by addition of DL-5-HTP 2.5 mg/kg instead of L-DOPA. After DL-5-HTP 2.5 mg/kg mount percentage and intromission percentage were significantly decreased and mount latency was increased. No significant effects were obtained on the other parameters recorded.

There were no signs of peripheral effects of the precursors among the subjects in these experiments.

The Effect of DL DOPS

DL-DOPS was given after the same combined pargyline + MK486 pretreatment as in the preceding experiment in the doses 3.0, 10 or 30 mg/kg. At none of these dose levels was there any facilitatory effect on any of the behavioral parameters recorded (table IV). At the highest dose tested 30 mg/kg mount latency was significantly increased and the number of mounts/min. was decreased. The predominant change of the overt behavior was a dose-dependent decrease of locomotor activity (see below). At even higher doses (60 mg/kg) the decrease in motor activity was still more pronounced.

Motor Activity

Pargyline 40 mg/kg increased motor activity (table V) while pargyline 20 mg/kg (1.5 hours) decreased motor activity when given either with or without MK486 although only the latter combined treatment induced a statistically significant difference. L-DOPA 10 mg/kg after pargyline alone and 2.5 mg/kg after pargyline + MK486 induced a significant further decrease in motor activity. An addition of DL-DOPS 30 mg/kg also caused a further decrease in motor activity while no further decrease in motor activity was

TABLE V The effect of certain drug treatments on motor activity in castrated male rats maintained on suboptimal response level with respect to mount percentage by means of weekly testosterone propionate treatment. One animal at a time was run for 10 min. in an Actimex activity meter beginning at zero hour. Statistical evaluation was performed with the Mann-Whitney U test.

MOTOR ACTIVITY					
Treatment	mg/kg	At hour	Counts/10 min	\pm	N
A1	Saline 0.4 ml	6	780	88	6
A2	Pargyline 40	6	1136	40	6
B1	Saline 0.4 ml	1.5 - 0.5	821	\pm 88	9
	Pargyline 20	1.5			
B2	Saline 0.4 ml	0.5	645	- 36	9
B3	+ DL-5-HTP 2.5	0.5	682	67	9
B4	L-DOPA 2.5	0.5	457	67	9
C1	Saline 0.4 ml	1.5 1.0 0.5	826	\pm 40	8
	Pargyline 20	1.5			
	MX486 50	1.0			
C2	Saline 0.4 ml	0.5	662	\pm 31	6
C3	DL-5-HTP 2.5	0.5	700	27	6
C4	L-DOPA 2.5	0.5	497	33	6
C5	DL-DOPA 10	0.5	610	+ 34	7
C6	DL-DOPA 30	0.5	515	\pm 48	6

Probabilities associated with the scores of the different treatment groups

Groups compared	p	Groups compared	p	Groups compared	p
A1 and A2	0.01	C1 and C2	0.01	C2 and C5	NS
B1 and B2	NS	C1 and C3	0.05	C2 and C6	<0.05
B1 and B3	NS	C1 and C4	0.001	C3 and C4	0.01
B1 and B4	<0.05	C1 and C5	0.01	C4 and C6	0.01
B2 and B3	NS	C1 and C6	0.001	C4 and C5	NS
B3 and B4	0.05	C2 and C3	NS	C5 and C6	NS
B3 and B4	NS	C2 and C4	0.05		

TABLE VI. Comparison of the effect of certain drug treatments on mount percentage and motor activity + increased, - decreased. NS = not significant ($p < 0.05$) one symbol $p < 0.05$ two symbols $p < 0.01$ three symbols $p < 0.001$ Statistics mount percentage = difference between pre-experimental and experimental test, motor activity = difference between drug and saline treated subjects.

DRUG TREATMENT	Mount Percentage	Motor Activity
A1 Paralyline 40 mg/kg		++
Paralyline 10 mg/kg		
B1 + Saline 0.4 ml	NS	NS
B2 + DL-5-HTP 2.5 mg/kg	--	NS
B3 + L-DOPA 10 mg/kg	NS	
Paralyline 20 mg/kg		
PK486 30 mg/kg		
C1 Saline 0.4 ml	NS	-
C2 DL-5-HTP 2.5 mg/kg		NS
C3 L-DOPA 2.5 mg/kg	+	-
C4 DL-DOPS 30 mg/kg	NS	

detected after addition of DL-5-HTP 2.5 mg/kg to either pre-treatment. For comparison between the effects of certain drug treatments on mount percentage and motor activity table VI was constructed. It is apparent that a change in one behavior is not associated with a corresponding change in the other behavior.

Scoring of the Orientation of Activity during Copulatory Tests

Each subject was given the score of 1 for each of the 3 min in which a certain orientation of activity was displayed for at least 5 hundredths of a min consecutively. The following classes of behavior were distinguished: Activity oriented 1 towards the female 2 towards the environment 3 towards the subject itself and 4 immobility. This procedure was developed during the course of this investigation and was only applied to the precursor experiments with the combined pargyline + MK486 pretreatment. The results are given in tables VII (pre-experimental scores) and VIII. Irrespective of subsequent mounting or non-mounting: female-oriented activity (enough to be scored) was displayed by all subjects during the first min of the test. Thus the change of scores for female-oriented activity only reflect the change obtained during the last two min. During these two min the scores for female oriented activity were decreased among DL-5-HTP treated non-mounters (but not in mounters). Environment-oriented activity was increased among DL-DOPS 30 mg/kg treated mounters but not significantly changed among other treatment categories. The only significant change of scores for self-oriented activity was a decrease among DL-5-HTP treated mounters. The occurrence of immobility was a conspicuous effect among L-DOPA treated subjects. Seventeen out of 39 L-DOPA treated subjects got a score for immobility during at least one min. in the exp. test whereas none of these subjects got any score in the pre-exp. test ($p < 0.001$). This immobility was characterized by periods of 10 - 25 hundredths of a min. during which the subjects stopped ongoing activity just standing still. It is of particular interest that a facilitation of the copulatory behavior could occur in spite of this immobility. In other experiments it was found that this kind of immobility was more pronounced after L-DOPA 5.0 mg/kg and less pronounced after L-DOPA 1.0 mg/kg than it was after L-DOPA 2.5 mg/kg as used in the present experiments.

TABLE VII. Orientation of activity during copulatory tests in castrated male rats maintained on a submaximal response level with respect to sexual percentage by means of weekly testosterone propionate treatment. The table shows the percentage distribution of pre-experimental scores. Each subject was given the score of 1 for each of the 3 min. in which certain behavior was displayed for at least 0.05 min. consecutively

Score	Percentage distribution of pre-experimental scores														
	Orientation toward						Immobility								
	female			environment			self								
	0	1	2	3	0	1	2	3	0	1	2	3			
Mounters (N 116)	0	3	19	78	36	33	22	6	1	21	22	53	100	0	0
Non-mounters (N 86)	0	59	33	8	1	7	55	37	24	49	22	5	100	0	0

TABLE VIII. The effect of monoline procedures (after paraffine and methyl pretreatment) on orientation of activity during exploratory tests. The table shows the number of subjects which in the experimental test received scores different from the one in the 1st test. Scoring technique see table VII and Methods

Treatment (mg/kg)	Orientation toward					
	female		environment		self	
	P		+ P		+ P	
	Direction χ^2 changed score 1		the exp test and the associated probability		Immobility	
Saline 0.4 ml monsters (N 36) non-monster (N 25)	7 4 NS	10 12 NS	9 10 NS	0 0 NS	0 0 NS	0 0 NS
	7 5 NS	7 7 NS	7 7 NS	0 0 NS	0 0 NS	0 0 NS
Paraffine 20 + ME486 50						
Saline 0.4 ml monsters (N 19) non-monster (N 9)	3 2 NS	4 7 NS	4 6 NS	0 0 NS	0 0 NS	0 0 NS
	0 3 NS	4 2 NS	4 0 NS	0 0 NS	0 0 NS	0 0 NS
DL-5-ary 2.5 monsters (N 13) non-monsters (N 12)	3 2 NS	3 2 NS	6 0 0.05	0 0 NS	0 0 NS	0 0 NS
	0 1 0.05	5 3 NS	5 5 NS	0 0 NS	0 0 NS	0 0 NS
+ L-DOPA 2.5 monsters (N 16) non-monsters (N 2)	2 2 NS	5 5 NS	2 5 NS	0 7 0.01	0 0 NS	0 0 NS
	1 0 NS	1 0 NS	0 2 NS	0 0 NS	0 0 NS	0 0 NS
DL-DOPA 3.0 monsters (N 7) non-monsters (N 13)	4 0 NS	1 4 NS	4 0 NS	0 0 NS	0 0 NS	0 0 NS
	2 2 NS	3 4 NS	3 3 NS	0 0 NS	0 0 NS	0 0 NS
+ DL-DOPA 10 monsters (N = 13) non-monster (N 16)	2 3 NS	4 4 NS	3 4 NS	0 1 NS	0 1 NS	0 1 NS
	5 2 NS	2 5 NS	3 4 NS	0 1 NS	0 1 NS	0 1 NS
+ DL-DOPA 30 monsters (N 12) non-monsters (N 9)	5 1 NS	1 0 0.05	6 3 NS	0 0 NS	0 0 NS	0 0 NS
	2 3 NS	2 2 NS	0 5 NS	0 0 NS	0 0 NS	0 0 NS

DISCUSSION

This investigation demonstrates that the two monoamine oxidase inhibitors pargyline and nialamide inhibit testosterone-activated heterosexual copulatory behavior in the castrated male rat. The specificity of this inhibitory effect is however difficult to evaluate as it was associated with evident effects on gross behavior. Similar inhibitory effects of monoamine oxidase inhibitors on copulatory behavior in the intact male rat have recently also been reported by Tagliamonte et al (1971) and Dewsbury et al (1972).

The mount and intromission percentages as well as the number of mounts/min. were significantly decreased by DL-5-HTP given after a dose of pargyline which alone did not influence the copulatory behavior. In contrast L-DOPA given in an analogous experiment did not inhibit the copulatory response although the motor activity was decreased more by that treatment than by DL-5-HTP. This suggests that the antagonistic effect on copulatory behavior by a high dose of pargyline and nialamide was predominantly due to an increased serotonergic tone. (In the following the term serotonergic tone will be used but the possibility exists that other metabolites of 5-HTP than serotonin might contribute to this inhibitory effect.) That the inhibitory effect of pargyline is due to an increased serotonergic tone is further supported by the fact that pretreatment with the serotonin-synthesis inhibitor p-chlorophenylalanine prevents pargyline from inhibiting copulatory behavior (Tagliamonte et al 1971; Malmåns and Meyerson 1971). If DL-5-HTP is added to the combined p-chlorophenylalanine + pargyline treatment the inhibitory effect of pargyline is re-established (Malmåns and Meyerson 1971). The experiments with the extracerebral decarboxylase inhibitor MK486 given in addition to the pargyline + precursor treatments demonstrate that central nervous rather than peripheral serotonin exerts the inhibitory effect. In these experiments the difference between the effects of DL-5-HTP

and L-DOPA on copulatory behavior became still more pronounced. In fact the effects of the two precursors were opposites to each other with respect to mount percentage, mount latency, intromission percentage and intromission latency.

With the testing procedure used in this investigation only a very small number of subjects normally achieve ejaculation within the time of a test session. It is therefore only possible to detect drug induced decreases of the ejaculation latencies. Ejaculation was achieved by a significant number of subjects after treatment with pargyline + MK486 + L-DOPA which indicates a facilitation of central nervous mechanisms involved in the triggering of ejaculation. This finding also suggests that the reduction of ejaculation latency by D-amphetamine reported by Butcher et al (1969) may at least in part be due to an increased central nervous catecholaminergic tone.

Although L-DOPA facilitates several components of the copulatory behavior after combined pargyline + MK486 pretreatment, efforts in this laboratory to obtain similar effects of L-DOPA after extracerebral decarboxylase inhibition but without pargyline pretreatment have as yet failed (unpublished). This failure is in accordance with recently reported results on the effect of L-DOPA after extracerebral decarboxylase inhibition on copulatory behavior in intact male rats (Ryppö et al 1971). Several reasons for this discrepancy can be proposed such as a displacement of serotonin by metabolites of the higher doses of L-DOPA necessary to obtain a similar increase in the catecholaminergic tone or a different distribution within the central nervous system of the newly formed catecholamines after pargyline pretreatment than without this pretreatment.

Regarding the specificity of the opposite effects of L-DOPA and DL-5-HTP on copulatory behavior as demonstrated in this investigation, motor activity measurements and scoring of the activity during copulatory tests give no reason to believe that these differences are merely secondary to changes of the general activity of the subjects. Since L-DOPA facilitated copulatory behavior

while causing significant reduction in motor activity and even periods of immobility it is obvious that there is a great degree of specificity with respect to the facilitatory effects on copulatory behavior. No such motor activity changes were detected after DL-5-HTP treatment in the doses used. Concerning the orientation of activity during copulatory tests it is of particular interest that the scores for female-oriented activity were significantly decreased among DL-5-HTP treated non-mounters. This finding indicates that other components of the male rat's interaction with the female than copulatory are also inhibited by DL-5-HTP.

Since small amounts of the precursors DL-5-HTP and L-DOPA were used in the present investigation and since opposite effects were obtained on the copulatory behavior displacement of catecholamines by serotonin or of serotonin by catecholamines should not be of any major significance. It is likely that the effects obtained by DL-5-HTP are due to an increased serotonergic tone and the effects obtained by L-DOPA due to an increase of the catecholaminergic tone.

In analogous experiments no facilitatory effects were seen after treatment with the noradrenaline precursor DL-DOPS. Possibly no functionally effective increase of the central nervous noradrenaline content was achieved. However, like L-DOPA DL-DOPS decreased motor activity. The absence of facilitatory effects after DL-DOPS and the increase in mount latency (in contrast to the decreased mount latency by L-DOPA) favours dopamine rather than noradrenaline as a neurotransmitter mediating a facilitation of testosterone-activated heterosexual copulatory behavior in the castrated male rat. A facilitatory function of dopamine is also suggested from other findings previously reported from this laboratory: Copulatory behavior was inhibited by the tyrosine hydroxylase inhibitor α -methyl-p-tyrosine but not by the dopamine β -hydroxylase inhibitor FLA-63 and it was facilitated by the dopamine receptor stimulating agent apomorphine but not by the noradrenaline receptor stimulating agent clonidine (Malmåa and Meyerson 1972; see also Malmåa 1973c, d).

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COPULATORY BEHAVIOR IN THE MALE RAT AFTER IMPAIRED MONOAMINERGIC NEUROTRANSMISSION

By

CARL OLOF MALMÖKS

ABSTRACT

The two monoamine depletors reserpine 0.5 mg/kg and tetrabenazine 1.0 and 3.0 mg/kg suppressed the testosterone-activated heterosexual copulatory behavior in castrated male rats. A decrease of the copulatory response was also seen after the catecholamine synthesis inhibitor α -methyl-p-tyrosine 75 and 150 mg/kg. In contrast the serotonin synthesis inhibitor p-chlorophenylalanine 4 x 100 mg/kg facilitated the response. The dopamine β hydroxylase inhibitor FLA-63 10 mg/kg did not decrease the copulatory response. The percentage of subjects which initiated copulatory behavior was decreased after the neuroleptics chlorpromazine 1.0 and 2.5 mg/kg and pimozide 0.25 mg/kg but not after the nor-adrenaline receptor blocking agent phenoxybenzamine 1.0 and 3.0 mg/kg or the adrenergic β -receptor blocking agent propranolol 1.0 and 4.0 mg/kg. It is suggested that testotosterone-activated heterosexual copulatory behavior in the castrated male rat is facilitated by decreased serotonin regulation and inhibited by decreased dopaminergic tone.

INTRODUCTION

Recent research has demonstrated that p-chlorophenylalanine (PCPA) facilitates components of heterosexual copulatory behavior in the intact (Sheard 1969; Tagliamonte et al. 1971; Salis and Desbary 1971; Ahlenius et al. 1971) and castrated (Malmöks and Meyerson

1971) male rat as well as male-to-male mounting in the intact (Tagliamonte et al 1969; Shillito 1970; Mitler et al 1972) and castrated (Gessa et al 1970; Malmöås and Meyerson 1971; Bond et al 1972) male rat. These behavioral effects are most likely due to the predominant effect of PCPA namely a decrease of the biosynthesis of serotonin (Roe and Weissman 1966; Jequier et al 1967)

In contrast to the great interest devoted to the effect of PCPA, little attention has been paid to the effect on copulatory behavior of drugs with a selective influence on catecholaminergic neurotransmission

The aim of the present investigation was to study the effect on copulatory behavior of drugs which induce a general or selective decrease of the monoaminergic tone and to evaluate the specificity of the effects obtained. Castrated male rats supplied with small amounts of testosterone propionate (TP) were used in order to avoid drug interactions with the gonadal hormone production and to be able to maintain a submaximal response level in the pre-drug state

MATERIAL AND METHODS

A procedure has been developed to maintain castrated male rats on a submaximal response level with respect to the percentage of subjects which displayed copulatory behavior (Malmöås 1973a). The method is also described in detail in Malmöås (1973b) from which no change has been made (See page 48 in this supplement)

Subjects

Experimental subjects were about 450 male Wistar rats castrated as adults. In one experiment bilaterally adrenalectomized castrated subjects were used which besides tap water also were supplied with 0.9% saline in their home cages and were injected once weekly with free dexamethasone 0.5 µg/animal given at the same day as TP

Injected materials

The hormones used were testosterone propionate (TP) estradiol benzoate progesterone and free dexamethasone (Organon). The neuropharmacological compounds used were reserpine (Serpasil^R Ciba ampoules 2.5 mg/ml) tetrabenazine HCl (Mitoman^R Hoffman-La Roche) dl-p-chlorophenylalanine methyl ester HCl (PCPA B69/17 Hissle) dl- α -methyl-p-tyrosine methyl ester HCl (oMT H44/68 Hissle) bis(4-methyl-1 homopiperazinylthiocarbonyl)-disulphide (FLA-63 H83/39 Hissle) chlorpromazine HCl (Hibernal^R Leo ampoules 25 mg/ml) pimozide (Janssen) phenoxybenzamine HCl (Dibenyline^R Smith Kline & French) and dl-propranolol HCl (Inderal^R ICI). The hormones were dissolved (or, dexamethasone suspended) in olive oil and the neuropharmacological agents dissolved or diluted in saline (except reserpine which was diluted with distilled water). Pimozide was dissolved in glacial acetic acid diluted with saline and the pH adjusted to 5 by addition of 2 N NaOH. Phenoxybenzamine was dissolved in hot acid propylene glycol (1% solution) and diluted with saline immediately before injection. Hormones were given subcutaneously while the neuropharmacological agents and saline were given intraperitoneally all in a volume of 1 ml/kg. (Exception: propranolol 4 mg/kg was given 1 ml/kg i.p. and 3 ml/kg s.c.) Doses mentioned in the text and tables always refer to the forms of the compounds stated above.

Three classes of neuropharmacological agents have been used: 1. Monoamine depletors (reserpine tetrabenazine) 2. Monoamine synthesis inhibitors (PCPA oMT FLA-63) and 3. Monoamine receptor blocking agents (chlorpromazine pimozide phenoxybenzamine and propranolol). Whereas oMT inhibits the catecholamine synthesis at the tyrosin hydroxylase level (Spetner et al 1965) FLA-63 inhibits the enzyme that converts dopamine to noradrenaline dopamine β hydroxylase (Florvall and Corrodi 1970). Chlorpromazine blocks both dopamine and noradrenaline receptors (Andén et al 1970) pimozide blocks dopamine but not noradrenaline receptors (Andén et al 1970) phenoxybenzamine blocks noradrenaline but not dopamine receptors (Andén et al 1966 1967 resp 1966a) while propranolol blocks adrenergic β -receptors (Black et al 1964).

RESULTS

The Effect of Reserpine

Thirty hours after reserpine 0.5 mg/kg the mount percentage and intromission percentage were significantly lower than in the pre-experiment (table IA). Furthermore mount latency was increased

and the number of mounts/min decreased. Changes of the other parameters did not reach statistical significance.

The subjects had an evident ptosis and a reduced motor activity. However, they displayed a normal non-copulatory interaction with the female (see below).

The Effect of Tetrabenazine

A significant decrease of mount percentage was obtained by tetrabenazine 10 and 30 mg/kg given 2 hours before the copulatory tests (table IB). Other parameters were not significantly changed.

The overt behavior was little affected by tetrabenazine 10 mg/kg but after 30 mg/kg the subjects sometimes stopped ongoing activity just standing still for short periods (0.10 - 0.30 min.) and had a decreased motor activity. However, the subjects displayed a normal amount of anogenital sniffing and pursuit of the female (see below). Neither ptosis nor hunched backs were noticed in these subjects.

The Effect of PCPA

PCPA 100 mg/kg was given either as a single dose 6 hours before the copulatory test or repeatedly for 4 consecutive days, the last injection 6 hours before the copulatory test. Six hours after PCPA 100 mg/kg neither mount percentage nor intromission percentage was significantly changed (table IIA). The only significant change of the copulatory parameters was a decreased mount latency. In contrast, when 100 mg/kg of PCPA was given for 4 consecutive days, a clearcut increase in mount and intromission percentages, a decreased mount latency and an almost significant ($p = 0.05$) increase in intromission ratio was seen 6 hours after the last injection. A striking effect of the PCPA treatment was the occurrence of ejaculations, whereas no subject ejaculated in the pre-exp

test 20 out of 39 subjects ejaculated in the exp test (13 out of 15 subjects which achieved intromission in the pre-exp test) None of the controls ejaculated The median ejaculation latency after PCPA treatment was 2.05 min The absolute number of mounts with or without intromission was not significantly different from the pre-exp test but they were achieved during a shorter time and thus at a faster rate

The main effect on the overt behavior was a decreased motor activity (see below)

The Effect of PCPA in Adrenalectomized Subjects

Since ACTH given subcutaneously brought about a facilitation of certain components of the male copulatory behavior in methodologically analogous experiments (see Malmk 1973a) it was of interest to study the effect of PCPA in adrenalectomized subjects PCPA 100 mg/kg was given one day followed by 50 mg/kg for 3 consecutive days the last injection 6 hours before the copulatory tests As is shown in table IIB PCPA induced a clearcut increase in mount and intromission percentages as well as in the number of mounts/min and there was a significantly decreased mount latency There were no ejaculations however

Among the PCPA treated subjects the gross appearance in spite of the dexamethasone treatment was affected: the subjects had tangled fur and hunched backs

The Effect of dMT

dMT 75 mg/kg given 6 hours before the copulatory tests induced a significant decrease in mount percentage and intromission percentage an increased mount latency and a decreased number of mounts/min. (table IIA) The other parameters were not significantly changed.

TABLE IIIA. The effect of aMT on copulatory behavior in castrated male rats maintained on subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. The drug and saline injections were given at 6 hours before the experimental tests. $M \pm$ median.

$\bar{p} = 0.05$ $b = p = 0.01$ $\bar{p} = 0.001$

Copulatory Parameter	Treatment mg/kg					
	A1			A2		
	Saline 0.4 ml			aMT 75		
	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>						
Percentage	59	56	34	60	37 ^b	35
Latency ^{MD}	18	13	14	15	80	13
Mo/min ^{MD}	3.0	2.7	14	3.0	1.7 ^b	13

<u>Intrusion</u>						
Percentage	41	35	34	46	9	35
Latency ^{MD}	15	20	9	10	40	3
Ratio ^{MD}	30	45	9	38	69	3

<u>Ejaculation</u>						
Percentage	0	0	34	0	0	35

No obvious effect on the overt behavior was seen (see below).

aMT 150 mg/kg was given 3 or 6 hours before the copulatory tests (table IIIB). This dose of aMT decreased mount percentage and in the 6 hour experiment also the intrusion percentage. The small number of mounting subjects in the exp. test does not permit an estimate of the effects on other copulatory parameters.

In the 3 hour experiment the locomotor activity was reduced but otherwise no obvious change of the overt behavior was seen. In the 6 hour experiment the subjects had a tendency to stop on-going activity standing still in a hunched back posture.

TABLE IV. The effect of oMT on copulatory behavior in oestrated male rats maintained on suboptimal reproductive level with respect to sexual performance by means of weekly testosterone propionate treatment. The drug and saline injections were given at 6 hours or 12 at 3 hours before the experimental tests. M: median
b p 0.01, p 0.001

Treatment t, mg/kg

Copulatory parameter	p1		p2		p3	
	Saline 0.4 ml		oMT 150		oMT 150	
	Pre-exp	Exp	N	Pre-exp	Exp	N
Percentage	43	45	47	53	16 ^b	22
Latency ^{Md}	33	40	15	15	65	4
No./min ^{Md}	3.2	2.5	15	3.9	1.7	4

Intromission

Percentage	38	38	47	32	14	22
Latency ^{Md}	50	60	11	20	80	3
Ratio ^{Md}	80	50	11	56	50	3

Ejaculation

Percentage	0	0	47	0	0	22

TABLE IV The effect of FLA-63 on copulatory behavior in castrated male rats maintained on a subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. The drug and saline treatments were given 4 hours before the experimental tests
Mdn median. p 0.05

Copulatory parameter	Treatment mg/kg					
	Saline 0.4 ml			FLA 63 10		
	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>						
Percentage	53	53	40	55	48	33
Latency ^{Mdn}	15	10	17	16	20	14
Mo/min ^{Mdn}	2.7	2.3	15	2.0	3.3	13

<u>Intromission</u>						
Percentage	35	35	40	24	16	33
Latency ^{Mdn}	30	20	9	45	40	6
Ratio ^{Mdn}	55	55	9	33	63	4

<u>Ejaculation</u>						
Percentage	0	5	40	6	3	33

The Effect of FLA-63

FLA-63 10 mg/kg given 4 hours before the copulatory tests had no inhibitory effect on the copulatory behavior (table IV). On the contrary the number of mounts/min was increased.

FLA-63 10 mg/kg decreased locomotor activity (see below) and the subjects had a slight but evident ptosis. At a higher dose (25 mg/kg) the subjects sat immobile crouched and did not take any notice of the female.

TABLE V The effect of chlorpromazine on copulatory behavior in oestrated male rats maintained on subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. The drug and saline injections were given 1 hour before the experimental tests. Md median. B $p < 0.05$ C $p < 0.001$

Copulatory parameter	Treatment mg/kg								
	Saline 0.4 ml			Chlorpromazine 1.0			Chlorpromazine 2.5		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>									
Percentage	49	44	57	58	36 ^b	50	53	10 ^c	30
Latency ^{Md}	20	19	23	20	10	18	50	30	2
No/min ^{Md}	2.7	3.0	23	2.5	3.0	18	3.2	2.2	2
<u>Intromission</u>									
Percentage	32	39	57	38	18	50	43	7 ^b	30
Latency ^{Md}	23	23	8	35	45	9	20	10	1
Ratio ^{Md}	61	61	8	93	67	9	67	84	1
<u>Ejaculation</u>									
Percentage	0	0	57	0	0	50	0	0	30

The Effect of Chlorpromazine

Chlorpromazine 1.0 mg/kg given 1 hour before the copulatory tests caused a slight but significant, decrease in mount percentage (table V). The decrease in mount percentage was more pronounced after chlorpromazine 2.5 mg/kg. This dose also decreased intromission percentage significantly. No other parameter was significantly changed.

The gross appearance was not affected by chlorpromazine 1.0 mg/kg. A slight ptosis and reduced locomotor activity was seen after 2.5 mg/kg (see below). Before the introduction of the female into the observation cage the subjects treated with chlorpromazine 2.5 mg/kg had a tendency to stand immobile. This tendency was no longer present after the introduction of the female.

TABLE VI The effect of pimozide on copulatory behavior in oestrated male rats maintained on a scheduled response level with respect to mount percentage by means of weekly testosterone propionate treatment. Drug and saline treatments were given 3 hours before the experimental test. MI median = p 0.05 ■ p 0.01 = 0.001

Copulatory parameters	Treatment mg/kg								
	Saline 0.4 ml			Pimozide 0.10			Pimozide 0.25		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>									
Percentage	54	54	41	53	50	40	55	21 ^c	42
Latency ^{MD}	18	25	16	25	18	18	15	25 ^b	9
Mo/MI ^{MD}	2.7	2.3	15	2.5	2.7	18	4.0	2.3	9
<u>Intrusion</u>									
Percentage	41	34	41	38	33	40	45	19	42
Latency ^{MD}	23	30	10	30	30	9	20	53	8
Ratio ^{MD}	61	50	10	64	38	9	70	52	8
<u>Ejaculation</u>									
Percentage	0	2	41	0	8	40	0	0	42

The Effect of Pimozide

Pimozide 0.10 mg/kg given 3 hours before the copulatory tests did not significantly change any of the copulatory parameters but 0.25 mg/kg significantly decreased mount percentage and intrusion percentage increased mount latency and decreased the number of mounts/min (table VI)

In contrast the overt behavior was unaffected: there was no prothesis or reduction in motor activity (see below)

TABLE VII. The effect of phenoxybenzamine on copulatory behavior in castrated male rats maintained on a subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. Drug and saline treatments were given 2 hours before the experimental test. Md = median.

Copulatory parameter	Treatment mg/kg								
	Saline 0.4 ml			Phenoxybenzamine 1.0			Phenoxybenzamine 3.0		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>	83	56	36	43	53	30	57	47	30
Latency ^{Md}	15	10	15	18	15	12	15	10	11
No./min ^{Md}	3.0	3.3	15	3.3	3.0	12	2.8	3.2	10
<hr/>									
<u>Emission</u>									
Percentage	42	44	36	40	33	30	47	37	30
Latency ^{Md}	30	20	13	83	80	8	20	20	8
Ratio ^{Md}	87	53	13	66	53	8	77	58	8
<hr/>									
<u>Ejaculation</u>									
Percentage	0	0	36	0	0	30	3	3	30

The Effect of Phenoxybenzamine

There was no significant effect on any of the copulatory parameters recorded after phenoxybenzamine 1.0 or 3.0 mg/kg given 2 hours before the copulatory tests (table VII).

The subjects had clearcut ptosis and reduced locomotor activity after phenoxybenzamine 3.0 mg/kg (see below) but not after 1.0 mg/kg.

At a still higher dose (10 mg/kg) the subjects were crouched immobile and did not take any notice of the female.

TABLE VIII The effect of propranolol on copulatory behavior in castrated male rats maintained on subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. Drug and saline treatments were given 45 min. before the experimental test. Md = median. b p 0.01 c p 0.001

Copulatory parameter	Treatment mg/kg								
	Saline 1.6 ml			Propranolol 1.0			Propranolol 4.0		
	Pre-exp	Exp	N	Pre-exp	Exp	N	Pre-exp	Exp	N
<u>Mount</u>									
Percentage	48	44	34	44	50	36	36	33	36
Latency ^{Md}	25	20	19	25	10	13	15	10	16
Ko/min ^{Md}	2.0	2.3	19	2.7	2.0	13	2.7	1.5 ^b	16
<u>Intrusion</u>									
Percentage	26	30	34	28	18	36	47	17 ^c	36
Latency ^{Md}	58	50	10	83	25	5	45	30	6
Ratio ^{Md}	54	58	10	60	22	5	57	25	6
<u>Ejaculation</u>									
Percentage	0	0	34	0	0	36	0	0	36

The Effect of Propranolol

There was no significant change in mount percentage after propranolol 1.0 or 4.0 mg/kg given 45 min. before the copulatory tests (table VIII). However, propranolol 4.0 mg/kg significantly decreased intrudmission percentage and the number of mounts/min. All subjects given propranolol had a decreased intrudmission ratio ($p < 0.01$ pooled data from both dosage groups).

The overt behavior was not influenced by propranolol in the doses used.

TABLE II. The effect of certain drug treatments on motor activity in castrated male rats maintained on subnormal response level with respect to mount percentages by means of weekly testosterone propionate treatment. One animal at a time was run for 10 min. in an Actimeter[®] activity meter beginning at zero hour. Statistical evaluation was performed with the Mann-Whitney U test. $\alpha = 0.05$
 $b = p < 0.01, c = p < 0.001$

MOTOR ACTIVITY

Treatment mg/kg	At hour	Counts/10 min ⁺	n.	N
Saline 0.4 ml	30	764 ⁺ 34	8	
Risperidine 0.5	30	521 ²² ^c	8	
Saline 0.4 ml	2	853 [±] 26	6	
Tetabenazine 1.0	2	718 ⁺ 45 ^b	6	
Tetabenazine 3.0	2	426 [±] 67 ^b	6	
Saline 4.0 0.4 ml	78 54 -30 -6	916 34	6	
PCPA 4 x 100	78 54 30 -8	336 [±] 62 ^b	6	
oMT 150	3	401 ⁺ 29 ^b	6	
Saline 0.4 ml	6	760 28	9	
oMT 75	6	668 [±] 54	9	
Saline 0.4 ml	4	871 [±] 19	7	
FlA-63 10	4	514 [±] 23 ^c	7	
Saline 0.4 ml	1	773 [±] 18	10	
Chlorpromazine 1.0	1	926 27 ^c	10	
Chlorpromazine 2.5	-1	680 ⁺ 19 ^a	7	
Phenoxymethamine 3.0	2	593 ⁺ 25 ^c	10	
Saline 0.4 ml	3	738 ⁺ 27	9	
Pimozide 0.25	3	684 [±] 27	9	
Saline 1.6 ml	0.75	801 [±] 58	6	
Propranolol 1.4 0	0.75	832 [±] 44	6	

TABLE X. Comparison of the effect of certain drug treatments on mount percentage and motor activity ++ increased, -- decreased. NS = not significant (p > 0.05) one symbol = p < 0.05 two symbols = p < 0.01 three symbols = p < 0.001 Statistics mount percentage = difference between pre-experimental and experimental test, motor activity = difference between drug and saline treated subjects

DRUG TREATMENT	Mount Percentage	Motor Activity
Reserpine 0.5 mg/kg		-
Tetrabenzine 1.0 mg/kg		
Tetrabenzine 3.0 mg/kg		
PCPA 4 x 100 mg/kg	++	
oMT 75 mg/kg		NS
oMT 150 mg/kg		
FLA-63 10 mg/kg	NS	
Chlorpromazine 1.0 mg/kg		+
Chlorpromazine 2.5 mg/kg		
Pimozide 0.25 mg/kg		NS
Phenoxylbenzamine 3.0 mg/kg	NS	
Propranolol 4.0 mg/kg	NS	NS

Motor Activity

Motor activity as measured by the Animex^R activity meter was significantly increased by chlorpromazine 1.0 mg/kg and not significantly changed by oMT 75 mg/kg pimozide 0.25 mg/kg and propranolol 4 mg/kg (table IX). Motor activity was decreased by all other drugs and doses tested (reserpine 0.5 mg/kg tetrabenzine 1.0 and 3.0 mg/kg PCPA 4 x 100 mg/kg oMT 150 mg/kg FLA-63 10 mg/kg chlorpromazine 2.5 mg/kg and phenoxylbenzamine 3.0 mg/kg). For comparison of the effect of the drugs on mount percentage and motor activity table X was constructed. It is apparent that a change

in one behavior is not necessarily associated with a corresponding change in the other behavior. It is of particular interest that the facilitatory effects of PCPA can occur in spite of a pronounced decrease in rotor activity.

Scoring of the Orientation of Activity during Copulatory Tests

Each subject was given the score of 1 for each of the 3 min. in which a certain orientation of activity was displayed for at least 0.05 min. consecutively. The following classes of behavior were distinguished: Activity oriented 1. towards the female, 2. towards the environment, 3. towards the subject itself and 4. immobility. The distribution of pre-experimental scores is given in table XI and the changes of scores after treatments are given in tables XII (reserpine and tetrabenazine), XIII (oMT and PIA-63), XIV (chlorpromazine and piroxide) and XV (phencyclamine and propranolol).

Irrespective of subsequent mounting or non-mounting, female-oriented activity (enough to be scored) was displayed by all subjects during the first min. of the test. Thus, the change of scores for female-oriented activity only reflects the change obtained during the last two of the 3 min. During these two min., the scores for female-oriented activity were significantly decreased among piroxide 0.25 mg/kg treated non-mounters. The scores for environment-oriented activity were significantly increased among oMT 75 mg/kg treated non-mounters and piroxide 0.25 mg/kg treated mounters and decreased among reserpine 0.5 mg/kg treated non-mounters. The scores for self-oriented activity were increased among chlorpromazine 1.0 mg/kg treated mounters. Tetrabenazine 3.0 mg/kg treated non-mounters received increased scores for immobility. No other significant change of the scores was obtained. Thus, a general feature of the compounds tested is that a dose sufficient to change the copulatory behavior has fairly small effects on the other behavior patterns recorded.

TABLE II Orientation of activity during copulatory tests in castrated male rats maintained on subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. The table shows the percentage distribution of μ immobility scores. Each subject was given the score of 1 for each of the 3 sds in which certain behavior was displayed for at least 0.05 min, consecutively

Percentage distribution of μ e-experimental scores

	Orientation toward												Immobility	
	female				environment				self					
Sec	0	1	2	3	0	1	2	3	0	1	2	3		
Mounters (N = 201)	0	2	19	80	18	37	18	10	2	13	18	11	99	1
Non-mounters (N = 319)	0	59	30	11	0	8	51	41	22	39	33	6	100	0

TABLE XII Orientation of activity during expulsive tests The table shows the number of subjects which, after treatment with saline or reserpine (A) resp. tetrabenazine (B) (exp. test) obtained scores different from the one in the $\mu = \mu_{\text{exp}}$ test. Scoring techniques: see table XI and Methods.

Treatment (mg/kg)	Orientation towards					self		Immobility		
	female		environment			test		nd the associated probability		
	Direction		f changed score in the exp			test		nd the associated probability		
	+	P	+	P	P	+	P	+	P	
A1 Saline 0.4 ml mounters (N = 11) non-mounters (N = 12)	0 4	1 2	MS MS	4 4	2 2	MS MS	3 2	1 5	MS MS	0 0
A2 Reserpine 0.5 mounters (N = 11) non-mounters (N = 16)	2 5	1 5	MS MS	5 11	3 1	MS 0.01	5 3	0 5	MS MS	0 0
B1 Saline 0.4 ml mounters (N = 14) non-mounters (N = 13)	3 5	1 3	MS MS	4 4	4 6	MS MS	2 3	3 4	MS MS	1 0
B2 Tetrabenazine 1.0 mounters (N = 16) non-mounters (N = 21)	5 8	2 6	MS MS	4 4	8 8	MS MS	6 5	2 6	MS MS	0 0
B3 Tetrabenazine 3.0 mounters (N = 4) non-mounters (N = 19)	0 2	0 5	MS MS	0 3	2 2	MS MS	1 11	0 4	MS MS	0 0

TABLE XIII Orientation of activity during exploratory tests. The table shows the number of subjects which after treatment with saline or oil (A) resp. FLA-63 (B) (resp. test) obtained score different from the one in the pre-experimental test. Scoring techniques see table XI and Methods

Treatment (mg/kg)	Orientation towards									
	female					environment				
	Direction of changed score in the					score in the				
	p					p				
	if					associated probability				
	p					p				
	immobility									
A1	Saline 0.4 ml									
	mounter (N 14)	0	3	N3	4	2	N6	2	2	N3
	non-mounters (N 8)	1	2	N3	3	3	N3	2	3	N5
A2	PC 75									
	mounter (N 13)	7	1	N3	2	9	N5	6	2	N5
	non-mounters (N 14)	3	8	N3	0	6	0.05	4	6	N3
B1	Saline 0.4 ml									
	mounter (N 13)	4	3	N3	6	4	N3	4	4	N3
	non-mounters (N 14)	3	3	N3	6	3	N3	3	4	N3
B2	FLA-63 11									
	mounters (N 13)	1	4	N3	4	4	N3	1	2	N3
	non-mounters (N 13)	3	5	N3	7	1	N3	6	4	N3

TABLE XIV Orientation of activity during exploratory tests The table shows the number of subjects which, after treatment with saline or chlorpromazine (A) resp. pimozide (B) (mp test) obtained score different from the one in the pre-explorational test. Scoring technique see table XI and Methods

Treatment (mg/kg)	Orientation towards						self		immobility			
	familiar		environment		self		immobility					
	Direction		score		t		score					
	P	P	+	-	P	P	-	P				
A1 Saline 0.4 ml mounter (N 22) non-mounters (N = 22)	3	5	MS	7	0	MS	6	9	MS	0	0	MS
	6	5	MS	5	0	MS	5	7	MS	0	0	MS
A2 Chlorpromazine 1.0 mounters (N 18) non-mounters (N 20)	2	2	MS	9	2	MS	1	0	0.05	0	1	MS
	4	6	MS	6	11	MS	6	9	MS	0	0	MS
A3 Chlorpromazine 2.5 mounter (N 2) non-mounters (N = 13)	1	0	MS	1	0	MS	1	0	MS	0	0	MS
	2	2	MS	2	7	MS	3	5	MS	0	0	MS
B1 Saline 0.4 ml mounters (N 19) non-mounters (N 13)	3	2	MS	5	6	MS	4	6	MS	0	0	MS
	1	3	MS	1	3	MS	4	3	MS	0	0	MS
B2 Pimozide 0.10 mounters (N 18) non-mounters (N 17)	3	1	MS	7	7	MS	5	6	MS	0	0	MS
	6	3	MS	2	6	MS	7	6	MS	0	0	MS
B3 Pimozide 0.25 mounters (N 9) non-mounters (N 18)	2	0	MS	0	7	0.05	4	0	MS	0	0	MS
	6	1	0.05	4	5	MS	3	11	MS	0	0	MS

TABLE XV Orientation of activity during copulatory tests The table shows the number of subjects which after treatment with saline or phenylbenzidine (A) resp. propenolol (B) obtained scores different from the one in the pre-treatment test. Recording techniques see table XI and Methods

Treatment (mg/kg)	Orientation toward											
	female		environment		self		immobility					
	DI action of charged sex		into		test and the		associated probability					
	p		+ p		p		+ p					
A1 Saline 0.4 ml mounter (N = 13) non-mounters (N = 12)	2	1	MS	2	5	MS	2	1	MS	0	0	MS
	6	3	MS	4	4	MS	3	5	MS	0	0	MS
A2 Phenylbenzidine, 1.0 mounter (N = 12) non-mounters (N = 13)	2	1	MS	5	4	MS	5	0	MS	0	1	MS
	3	3	MS	2	3	MS	4	2	MS	0	1	MS
A3 Phenylbenzidine, 3.0 mounter (N = 10) non-mounters (N = 10)	2	1	MS	3	2	MS	3	1	MS	0	3	MS
	2	2	MS	3	2	MS	2	5	MS	0	2	MS
B1 Saline 1.6 ml mounter (N = 19) non-mounters (N = 22)	4	7	MS	5	6	MS	7	5	MS	0	0	MS
	3	3	MS	5	5	MS	4	6	MS	0	0	MS
B2 Propenolol 1.1 mounter (N = 13) non-mounters (N = 15)	5	5	MS	4	4	MS	4	1	MS	0	0	MS
	4	3	MS	4	4	MS	2	6	MS	0	0	MS
B3 Propenolol 1.4 mounter (N = 16) non-mounters (N = 13)	5	1	MS	4	6	MS	7	2	MS	0	0	MS
	1	1	MS	1	4	MS	4	6	MS	0	0	MS

DISCUSSION

The two monoamine depletors reserpine and tetrabenazine suppressed testosterone-activated heterosexual behavior in the castrated male rat. The specificity of this effect is suggested by the fact that the non-copulatory female-oriented activity which requires intact motor capabilities was not decreased. Abolishment of copulatory behavior in intact male rats by tetrabenazine (5 mg/kg) has previously been reported by Butcher et al. (1969). From the description of the overt behavior of their subjects it is obvious that they lacked the motor capabilities necessary for copulatory behavior to be displayed. Facilitatory effects on certain components of male copulatory behavior of reserpine (Soulaireac 1963; Dewsbury and Davis 1970) and tetrabenazine (Dewsbury 1972) have also been reported. Since intact rats were used in these studies and other parameters recorded, direct comparison with the present data cannot be made.

After repeated PCPA treatment there was an increased mount and intromission percentage, a decreased mount latency, an increased number of mounts/min., and an increased ejaculation percentage. With the exception of ejaculation percentage, the same effects were obtained both in non-adrenalectomized and adrenalectomized subjects. Thus, these effects on copulatory behavior were not secondary to an altered endogenous steroid hormone production.

Contrary to the effects of PCPA on the copulatory behavior, oMT had clearcut inhibitory effects: the mount and intromission percentages were decreased, the mount latency increased and the number of mounts/min. decreased. Notably, oMT 75 mg/kg did not significantly reduce motor activity while a pronounced decrease of motor activity was obtained by the repeated PCPA treatment.

When the data on selective serotonin (PCPA) and catecholamine (oMT) synthesis inhibition are compared, it is obvious that the effects were opposite to each other with respect to mount and intromission percentages, mount latency and the number of mounts/min.

Since catecholamine but not serotonin depletion was inhibitory these data suggest that reserpine and tetrabenazine inhibited components of the male copulatory behavior mainly by impairing catecholaminergic neurotransmission.

FLA-63 10 mg/kg decreased motor activity but copulatory behavior was not inhibited. In contrast to the decreased number of mounts/min after α MT 75 mg/kg FLA-63 increased the number of mounts/min. The difference in effect between α MT and FLA-63 indicates the significance of dopamine rather than noradrenaline for male copulatory behavior. This is further suggested by the effects of the receptor blocking agents.

Pimozide which in the doses used blocks dopamine but not noradrenaline receptors brought about inhibitory effects in a dose which did not decrease motor activity or otherwise disturb the overt behavior: mount and intromission percentages were decreased and there was an increased mount latency and a decreased number of mounts/min. Furthermore the scores for female-oriented activity were significantly decreased among pimozide treated non-mounters. This finding indicates that pimozide not only inhibits copulatory behavior but also other components of the male's interaction with the female.

Phenoxylbenzamine which is considered to block noradrenaline receptors but not dopamine receptors centrally and α -receptors peripherally decreased motor activity but did not inhibit copulatory behavior. In contrast chlorpromazine which blocks both noradrenaline and dopamine receptors induced a decrease of the mount and intromission percentages.

Propranolol which blocks adrenergic β -receptors did not decrease mount percentage but decreased intromission percentage, intromission ratio and the number of mounts/min. Further experimentation is needed to elucidate the relationship between these effects and a blockade of adrenergic β -receptors. Notably propranolol was the only compound tested which changed intromission percentage without affecting mount percentage.

The data of the present investigation show that decreased catecholaminergic tone inhibits copulatory behavior in the male rat. Provided the noradrenergic tone was not more than moderately decreased no inhibition of the behavior was brought about; certain components were even facilitated (such as an increased number of mounts/min after FLA-63). If the noradrenergic tone was decreased to the extent that there were profound effects on the overt behavior copulatory behavior was inhibited. In contrast a drug-induced decrease of the dopaminergic tone inhibited copulatory behavior already in doses which produced little or no alteration of the overt behavior.

In methodologically analogous experiments it was recently found in this laboratory that an increase of the central nervous catecholamine content by means of L-DOPA, given after a combined treatment with a monoamine oxidase inhibitor (pargyline) and an extracerebral decarboxylase inhibitor (MW486) facilitated several components of the copulatory behavior while an increase of the serotonin content by means of addition of DL-5-HTP had essentially the opposite effect (Malenka and Meyerson 1972; Malenka 1973b). No facilitation of copulatory behavior was obtained by the selective noradrenaline precursor DL-DOPS. When considering the results of the present investigation in relation to the ones with monoamine precursors it appears that a decreased central nervous serotonergic tone facilitates a decreased dopaminergic tone inhibits while a decreased noradrenergic tone has no specific inhibitory effect on ~~male~~ activated heterosexual copulatory behavior in the castrated male rat.

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EFFECTS OF LSD-25 CLONIDINE AND APOMORPHINE ON COPULATORY BEHAVIOR IN THE MALE RAT

By

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ABSTRACT

The effects of lysergic acid diethylamide (LSD) clonidine and apomorphine on testosterone activated heterosexual copulatory behavior in castrated male rats were studied. The percentage of subjects which displayed copulatory behavior was decreased by LSD 30 and 100 µg/kg while this percentage was increased after apomorphine 30 100 and 300 µg/kg. The facilitatory effects of apomorphine 100 µg/kg were blocked by pimoside 100 µg/kg. Clonidine 3 10 and 30 µg/kg did not change the percentage of subjects that displayed copulatory behavior. The results are discussed in terms of the possible relation to monoaminergic mechanisms involved in the regulation of certain components of male sexual behavior.

INTRODUCTION

Accumulating evidence suggests that lysergic acid diethylamide (LSD) mimics and/or facilitates the effect of serotonin on central nervous serotonergic receptors (Freedman 1961; Curtis and Davis 1962; Rosecrans et al 1967; Andén et al 1968; Diaz et al 1968; Aghajanian et al 1968 1970 1972; Lin et al 1969; Schurbert et al 1970; Eliasson et al 1972; for review see Aghajanian 1972a b). There is also evidence that clonidine has noradrenaline-like effects in the central nervous system (Andén et al 1970; Schmitt et al 1971; Broekamp and van Rossum 1972). Furthermore

increasing evidence indicates that apomorphine mimics and/or facilitates the effects of dopamine in the central nervous system (Ernst 1967; Andén et al. 1967; Roos 1969; Ungerstedt et al. 1969; Ungerstedt 1971)

This investigation deals with the effects of LSD, clonidine and apomorphine on testosterone-activated copulatory behavior in the castrated male rat. These drugs were used as tools in order to further elucidate the neural mechanisms involved in the regulation of this behavior.

MATERIAL AND METHODS

A procedure has been developed to maintain castrated male rats on a submaximal response level with respect to the percentage of subjects which displayed copulatory behavior (Malmeåke 1973a). The method is also described in detail in Malmeåke (1973b) from which no change has been made. (See page 48 in this supplement)

Subjects

Experimental subjects were about 195 male Wistar rats castrated as adult.

Injected materials

The hormones used were testosterone propionate (TP), estradiol benzoate and progesterone (Organon). The neuropharmacological compounds used were D-lysergic acid diethylamide tartrate (LSD, Sandoz, Basel), clonidine HCl (CH Boehringer Sohn, Ingelheim am Rhein), apomorphine HCl (Sandoz, Basel) and pimozide (Janssen, Beerse). The hormones were dissolved in olive oil and the neuropharmacological agents (except pimozide) in saline; pimozide was dissolved in a few drops of glacial acetic acid diluted with saline and the pH adjusted to 5 by addition of 2 N NaOH. All injections of hormones were given subcutaneously while the neuropharmacological agents and blank solution were given intraperitoneally all in a volume of 1 ml/kg. Do as mentioned in the text and tables always refer to the formulae of the compounds stated above.

The time from injection of LSD, clonidine and apomorphine to the onset of the copulatory tests was based on pilot experiments in which recordings of the overt behavior were made every six min. from injection.

RESULTS

The Effect of LSD

LSD 10 30 or 100 $\mu\text{g/kg}$ was given 12 min before the onset of the copulatory tests. The time 12 min was based on pilot experiments which showed that the effects on the overt behavior of LSD 300 $\mu\text{g/kg}$ (side-movements of the head hyperextensions of hind-legs tremor) had reached its maximum at that time.

LSD 10 $\mu\text{g/kg}$ did not significantly change any of the copulatory parameters recorded (table I). After 30 and 100 $\mu\text{g/kg}$ of LSD mount and intromission percentages were significantly decreased. After 30 $\mu\text{g/kg}$ of the drug there was also a significant decrease of the number of mounts/min. This effect was not possible to evaluate after 100 $\mu\text{g/kg}$ since too few subjects displayed mounting in the exp. test. No other copulatory parameters recorded were significantly changed.

The overt behavior was unaffected by the two lower doses of LSD tested. A few subjects showed side-movements of the head and intermittent hyperextensions of the hindlegs after LSD 100 $\mu\text{g/kg}$.

The Effect of Clonidine

Clonidine 3 10 or 30 $\mu\text{g/kg}$ was given 12 min before the onset of the copulatory tests. Pilot experiments showed that pronounced sedation with immobility was fully developed at that time after clonidine 100 $\mu\text{g/kg}$.

There was no significant change of the copulatory parameters recorded after clonidine 3 or 10 $\mu\text{g/kg}$ (table II). Clonidine 30 $\mu\text{g/kg}$ however significantly decreased mount latency as well as the number of mounts/min. No other copulatory parameter was significantly changed.

The overt behavior was not affected by clonidine 3 $\mu\text{g/kg}$. Locomotor activity was slightly decreased after 10 $\mu\text{g/kg}$ and there

TABLE II. The effect of clonidine on copulatory behavior in castrated male rats maintained on submaximal response level with respect to mount percentage by means of weekly testosterone propionate treatment. The drug and saline treatments were given 12 min before the experimental tests. Hd = hidden. P = 0.05.

Copulatory parameter	Treatment 10 µg/kg				Clonidine 10				Clonidine 10			
	P	Pre-exp	Exp	N	P	Pre-exp	Exp	N	P	Pre-exp	Exp	N
<u>Mount</u>												
Percentage	33	37	30		33	43	30		45	45	31	
Latency ^{Hd}	15	10	13		10	10	11		10	10	11	
Mo/min ^{Hd}	22	23	12		20	27	10		17	13	11	
Y transmission												
Percentage	37	40	30		33	17	30		29	23	31	
Latency ^{Hd}	20	25	7		30	13	2		23	30	4	
Ratio ^{Hd}	57	35	7		38	59	2		42	45	4	
<u> Ejaculation</u>												
Percentage	0	3	38		0	3	38		0	0	31	

Percentage 3 0 31 0 0 31 3 0 29

was a more pronounced decrease after clonidine 30 $\mu\text{g/kg}$. At higher doses (100 $\mu\text{g/kg}$) the subjects sat immobile and did not take any notice of the female.

The Effect of Apomorphine

Apomorphine 10, 30, 100 or 300 $\mu\text{g/kg}$ was given 12 min. before the onset of the copulatory tests. According to pilot experiments stereotyped gnawing, sniffing and licking were fully developed at 12 min. after apomorphine 100 $\mu\text{g/kg}$.

Mount percentage was significantly increased after apomorphine 30, 100 and 300 $\mu\text{g/kg}$ (table III). After apomorphine 100 $\mu\text{g/kg}$ there was also a significant increase in intromission percentage. Mount latency was significantly decreased after apomorphine 30 and 100 $\mu\text{g/kg}$. The number of mounts/min. was not changed by apomorphine 10 $\mu\text{g/kg}$, significantly increased by apomorphine 30 $\mu\text{g/kg}$, not significantly changed by apomorphine 100 $\mu\text{g/kg}$ and significantly decreased by apomorphine 300 $\mu\text{g/kg}$. The decline of the number of mounts/min. with increasing dose of apomorphine was possibly due to the increase of stereotyped motor patterns elicited by the higher doses.

All subjects treated with apomorphine 300 $\mu\text{g/kg}$ showed some kind of stereotyped behavior, ranging from gentle sniffing of the floor to compulsive licking, gnawing, chewing of wooden shavings from the floor and scratching on the walls of the observation cage. Sniffing of the floor and environment was the only stereotyped behavior displayed by subjects treated with apomorphine 100 $\mu\text{g/kg}$. No obvious stereotypies were seen after apomorphine 30 or 10 $\mu\text{g/kg}$.

The Effect of Apomorphine after Pimozide Pretreatment

Pimozide is a neuroleptic which blocks dopamine but not noradrena-

line receptors (Andén et al. 1970a). This drug (0.25 mg/kg) has been found to inhibit copulatory behavior as measured by the method used in this investigation (Hakala 1973c). In the present experiment pimozide was given in a dose found to have no detectable effects on the copulatory parameters recorded.

Pimozide 100 µg/kg was given 3 hours and apomorphine 100 µg/kg 12 min before the copulatory tests. All the effects on the copulatory behavior obtained by apomorphine 100 µg/kg were prevented by pimozide 100 µg/kg (table IVB).

The overt behavior was unaffected by this treatment regimen. No subject showed any stereotyped sniffing of the floor or environment as was seen when this dose of apomorphine was given alone.

Motor Activity

Motor activity as measured by the Animex^R activity meter was decreased by clonidine 30 µg/kg and by apomorphine 100 µg/kg while it was not significantly changed by LSD 30 µg/kg (table V). Pretreatment with pimozide 100 µg/kg blocked the apomorphine-induced decrease in motor activity. For comparison between the effect of certain drug treatments on mount percentage and motor activity table VI was constructed. It is apparent that a change in one behavior is not associated with a corresponding change in the other behavior.

Scoring of the Orientation of Activity during Copulatory Tests

Each subject was given the score of 1 for each of the 3 min. in which a certain orientation of activity was displayed for at least 0.05 min consecutively. The following classes of behavior were distinguished: Activity oriented 1 towards the female, 2 towards the environment, 3 towards the subject itself and 4 immobility. The results are given in tables VII (pre-exp. scores), VIII (LSD)

TABLE V The effect of certain drug treatments on motor activity in castrated male rats maintained on subnormal response level with respect to mount percentage by means of weekly testosterone propionate treatment. One animal at a time was run for 6 min. in an Actam[®] activity meter starting at zero min. Statistical evaluation was performed with the Mann-Whitney U test.

MOTOR ACTIVITY

Treatment	µg/kg	At min	Counts/6 min	s.e.m.	N
A1 Saline	0.4 ml	12	529	23	6
A2 LSD	30	12	544	27	6
A3 Clonidine	30	12	374	30	6
A4 Apomorphine	100	~ 12	426 ± 28		6
B1 Saline	0.4 ml	180 - 12	491 ± 18		6
B2 Pimozid	100	180			
Saline	0.4 ml	12	502	10	6
B3 Pimozid	100	180			
Apomorphine	100	12	476 ± 31		6

Probabilities associated with the comparison of the difference between treatment groups

Groups compared	P	Groups compared	P
A1 and A2	NS	B1 and B2	NS
A1 and A3	0.001	B1 and B3	NS
A1 and A4	0.001	B2 and B3	NS
A2 and A3	0.01		
A2 and A4	0.05		
A3 and A4	NS		

TABLE VI Comparison between the effect of certain drug treatments on mount percentage and motor activity. Increased, ↑ = decreased, ↓ = decreased. NS = not significant (p > 0.05) one symbol p < 0.05 two symbols = p < 0.01, three symbols = p < 0.001. Statistics: mount percentage = difference between pre-experimental and experimental test, motor activity = difference between drug and saline treated subjects

DRUG TREATMENT	Mount Percentage	Motor Activity
LSD 30 µg/kg		NS
Clonidine 30 µg/kg	NS	
Apomorphine 100 µg/kg	+	
Pimozid 100 µg/kg	NS	NS
Pimozide 100 µg/kg +		
Apomorphine 100 µg/kg	NS	NS

TABLE VII Orientation of activity during copulatory tests in castrated male rats maintained on subnormal response level with respect to round percentage by means of weekly testosterone propionate treatment. The table shows the percentage distribution of pre-experimental scores. Each subject was given the score of 1 for each of the 3 s.d.s. in which certain behavior was displayed for at least 0.55 s.d.s. consecutively

Percentage distribution of pre-experimental scores

	Orientation towards												Immobility			
	female				environment				self							
	0	1	2	3	0	1	2	3	0	1	2	3				
Score	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Mounters (N 229)	0	5	23	72	21	38	28	14	5	23	28	44	100	0	0	0
Non-mounters (N 225)	0	56	33	11	1	11	52	36	20	36	37	8	100	0	0	0

TABLE VIII The effect of LBD on orientation of activity during exploratory tests. The table shows the number of subjects which in the experimental test received a score different from the one in the spontaneous test. Scoring technique see table VII and Methods.

Treatment ($\mu\text{g/kg}$)	Orientation towards				self		immobility		
	familiar		environment						
	Direction of		hanged arc		in the exp		test and the		
	P		P		+ P		+ P		
Saline 0.4 ml mounters (N 13) non-mounters (N 13)	3	0 NS	3	4 NS	3	2 NS	0	0 NS	
	5	4 NS	2	4 NS	3	3 NS	0	0 NS	
LBD 10 mounters (N 14) non-mounters (N 20)	2	2 NS	5	4 NS	5	2 NS	0	0 NS	
	4	5 NS	5	4 NS	6	0 NS	0	0 NS	
LBD 30 mounters (N 10) non-mounters (N 16)	4	1 NS	1	7 NS	6	1 NS	0	0 NS	
	8	0 0.01	8	6 NS	9	0 0.01	0	0 NS	
LBD 100 mounters (N = 3) non-mounters (N 9)	1	1 NS	1	1 NS	1	0 NS	0	2 NS	
	3	0 NS	0	7 0.05	4	1 NS	0	1 NS	

Table II The effect of piraridine and piraridine apomorphine on orientation of activity during copulatory tests. The table shows the number of subjects which in the experimental test received scores different from the one in the pre-experimental test. Scoring techniques see table VII and Methods

Treatment ($\mu\text{g/kg}$)	Ori station toward				Immobility			
	female		environment		self		+ P	
	Direction of changed score		in th exp		test and the associated probability		+ P	
	P		+	P	+	P	+	P
A1 Saline 0.4 ml mounters (N 13) non-mounters (N 13)	3 2 MS 1 3 MS		5 6 MS 1 3 MS		4 6 MS 4 3 MS		0 0 MS 0 0 MS	
A2 Pimoridine 100 mounters (N 18) non-mounters (N 17)	3 1 MS 6 3 MS		7 7 MS 2 6 MS		5 6 MS 7 6 MS		0 0 MS 0 0 MS	
B1 Saline 0.4 ml mounters (N 14) non-mounters (N 13)	2 4 MS 4 2 MS		2 3 MS 3 3 MS		6 4 MS 9 5 MS		0 0 MS 0 0 MS	
B2 Pimoridine 100 Apomorphine 100 mounters (N 14) non-mounters (N 15)	4 2 MS 3 6 MS		5 6 MS 5 1 MS		6 3 MS 6 7 MS		0 0 MS 0 0 MS	

IX (clonidine) X (apomorphine) and Z (morphine) -
A certain amount of female-oriented activity was displayed by all subjects during the first two min. change of scores for female-oriented activity - change obtained during the last two of the two - two min. the scores for female-oriented activity among LSD 30 µg/kg treated non-mounters and morphine 30 and 100 µg/kg treated non-mounters and morphine 30 µg/kg treated mounters. LSD mounters got increased scores for activity while apomorphine 30 µg/kg treated non-mounters for this orientation of activity. Self-orientation increased among LSD 30 µg/kg treated non-mounters. Immobility were increased in clonidine as well as among apomorphine 300 µg/kg mounters. No other statistically significant differences for the different orientations of activity.

DISCUSSION

The results of the present investigation of clonidine and apomorphine had effects on testosterone-induced copulatory behavior in the castrated male rat. It affected copulatory behavior in different ways. The probable mechanisms of action of these drugs are not clear. The results will be discussed in relation to the results found in methodologically analogous experiments on copulatory behavior of other animals. The effects on copulatory behavior of other drugs on the same neurotransmission (cf. Malmberg and Malmberg, 1973b, c).

Table XI The effect of picroside and picroside apomorphine on orientation of activity during exploratory tests. The table shows the number of subjects which in the experimental test received a score different from the one in the pre-experimental test. Scoring technique see table VII and Methods

Orientation towards										
Treatme t (mg/kg)	female		environment		self		Immobility			
	Direction of changed score in th		exp		test and the associated probability					
	p		p		p		+ p			
A1 Saline 0.4 ml mounters (N 13) non-mounters (N 13)	3	2	NS	5	6	NS	4	6	NS	0 0 NS
	1	3	NS	1	3	NS	4	3	NS	0 0 NS
A2 Picrosid 100 mounters (N 18) non-mounters (N 17)	3	1	NS	7	7	NS	5	6	NS	0 0 NS
	6	3	NS	2	6	NS	7	6	NS	0 0 NS
B1 Saline 0.4 ml mounters (N 14) non-mounters (N 15)	3	4	NS	2	3	NS	6	4	NS	0 0 NS
	4	2	NS	3	5	NS	9	5	NS	0 0 NS
B2 Picrosid 100 Apomorphine 100 mounters (N 14) non-mounters (N 15)	4	2	NS	5	6	NS	6	3	NS	0 0 NS
	3	6	NS	5	1	NS	6	7	NS	0 0 NS

ing agent phencybenzamine did not affect mount or intromission percentage in analogous experiments but increased the number of mounts/min. (FIA-63) Thus neither compounds which increase nor compounds which decrease the noradrenergic tone had any significant influence on the percentage of subjects which displayed pre-ejaculation copulatory behavior provided that the doses used did not too seriously disturb the overt behavior of the subjects. However other components of the copulatory behavior (e.g. the number of mounts/min) seem to be more influenced by the noradrenergic tone.

Apomorphine

Apomorphine increased mount percentage and intromission percentage and decreased mount latency. The number of mounts/min was increased after 30 $\mu\text{g/kg}$ but decreased after 300 $\mu\text{g/kg}$ (possibly due to the increase in stereotyped motor patterns). Previously it was found that L-DOPA (given after pargyline and MK486 pretreatment) increased mount and intromission percentages and decreased mount latency. Thus L-DOPA and apomorphine facilitated components of the copulatory behavior in spite of the fact that both compounds decreased motor activity as measured by the Actimex^R activity meter. The facilitatory effects of apomorphine on copulatory behavior were blocked by a dose of pimozide which by itself had no significant effect on copulatory behavior. This dose of pimozide also blocked the facilitatory effects of L-DOPA on mount and intromission percentages (to be published). A higher dose of pimozide as well as treatment with the tyrosine hydroxylase inhibitor α -methyl-p-tyrosine decreased mount and intromission percentages increased mount latency and decreased the number of mounts/min. These inhibitory actions were not associated with a decrease in motor activity or other disturbances of the overt behavior. Notably pimozide in a dose which inhibited copulatory behavior decreased the scores for female-oriented activity among non-mounters.

In contrast these scores were increased after apomorphine 30 and 100 µg/kg. These findings suggest that an increased dopaminergic tone facilitates and a decreased dopaminergic tone inhibits both copulatory and non-copulatory components of the male rat's interaction with the female. Current data indicate the opposite relationship for drugs affecting the serotonergic tone.

Some studies on the effect of LSD and apomorphine on copulatory behavior in the intact male rat have previously been made. Facilitatory effects of LSD on certain components of the established copulatory behavior (Gillett 1960; Soulairec 1963; Bignami 1966) as well as inhibitory ones (Bignami 1966; Ujeno 1967) have been reported. Both facilitatory and inhibitory effects of a fairly high dose of apomorphine (0.8 mg/kg) have been demonstrated by Butcher et al. (1969). Since vigorously copulating intact subjects were used in these studies and other parameters recorded, meaningful comparisons with the present results are difficult to make.

Taken together the data of the present investigation show that LSD decreased the percentage of subjects which displayed pre-ejaculation copulatory behavior. In contrast this percentage was increased by apomorphine while clonidine had no effect on this behavioral component. When these data are compared with the ones obtained with other neuropharmacological agents (see above) it is suggested that the effects of LSD and apomorphine (on the percentage of subjects which displayed pre-ejaculation copulatory behavior) were due to functionally increased central nervous serotonin and dopamine receptor activity respectively.

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MONOAMINEPGIC INFLUENCE ON TESTOSTERONE-ACTIVATED COPULATORY BEHAVIOR IN THE CASTRATED MALE RAT

GENERAL SUMMARY AND CONCLUSIONS

This summary is based on the following communications: Malenka's 1973a (I) 1973b (II) 1973c (III) and 1973d (IV) The four papers will be referred to by the Roman numerals

THE METHOD (I)

Copulatory behavior in the male rat consists of a series of mounts with or without penile intrusion into the female vagina terminated by ejaculation This behavior is dependent on gonadal hormones; it disappears gradually after castration and can be restored to the pre-castration level by testosterone propionate (TP) (Shapiro 1937; Stone 1939; Beach and Holt-Tucker 1949) Most likely testosterone activates copulatory behavior in the male rat by a direct action on the central nervous system (Davidson 1966; Lisk 1967)

The main aim of the experiments presented in paper (I) was to develop a procedure appropriate for pharmacological studies on testosterone-activated heterosexual pre-ejaculation copulatory (mounting) behavior in the castrated male rat. One major objective was to find a schedule of TP treatment that induced and could maintain a submaximal response with respect to the percentage of subjects that displayed mounting (mount percentage in this summary also called copulatory response)

A testing procedure was used in which the male was given 5 min of adaptation to the observation cage before a sexually receptive female was introduced. The time allowed from the introduction of the female to the first mount was restricted to 3 min. If the male mounted the female within 3 min, the test continued for another 3 min, counted from the first mount, or until ejaculation occurred, whichever came first. Although the method was primarily designed to measure mount percentage, other components of the copulatory behavior were also recorded such as the percentages of subjects which achieved intromission and ejaculation, the latencies to the first mount and first intromission, the average number of mounts/min, and the percentage of mounts with intromission of the total number of mounts (intromission ratio). Tests were carried out regularly once weekly.

Within 5 weeks from castration the mount percentage had decreased from 100 to 50%. In non-mounting subjects, mounting behavior appeared 30-54 hours after a single injection of TP, irrespective of the dose given (0.10 - 0.80 mg/kg). The duration of the effect of TP on mount percentage was dose-dependent. Counted from the day of injection, the duration was 5-10 days after a dose of TP that induced a submaximal response (0.10 - 0.20 mg/kg), while after a supramaximal dose of TP (0.40 mg/kg) the duration was longer.

A schedule of weekly TP injections of 0.20 mg/kg or less was found to induce and maintain a submaximal response with respect to mount percentage. The response was dose-dependent and remained on a stable submaximal level for several months. Even after several months of weekly TP treatment, the response was sensitive to a change in the amount of TP given.

The TP-activated copulatory response was not changed by adrenalectomy. However, ACTH increased the copulatory response. This effect was likely caused by increased adrenal steroid hormone production, as no effect of ACTH was seen in adrenalectomized subjects. These data demonstrate that although the adrenals did not influence the TP-activated response under ordinary conditions, it was

possible to increase the response by stimulating the adrenals

A procedure to score the specific orientation of activity was developed as a tool to differentiate specific from non-specific effects of drugs on male copulatory behavior. It was found that irrespective of subsequent mounting or non-mounting all males approached and explored the female when she was introduced into the observation cage. A negligible amount of time during copulatory tests was devoted to immobility.

Previously neuropharmacological studies on heterosexual copulatory behavior in the male rat have exclusively been conducted in intact as a rule vigorously copulating subjects. Compared to previous procedures the present method offers the advantage of being independent of drug-effects on the gonadal hormone production. Furthermore, the copulatory response can be maintained at a submaximal level which permits a drug-induced increase or decrease of the response to be detected.

NEUROPHARMACOLOGICAL EXPERIMENTS

Increase of the Monoaminergic "Tone" (II)

A general increase of the monoaminergic tone achieved by the monoamine oxidase inhibitors pargyline (40 mg/kg) and nialamide (250 mg/kg) induced a decrease of mount percentage. A similar decrease of mount percentage was obtained by the serotonin precursor DL-5-HTP (1.0 and 2.5 mg/kg) given after pretreatment with a subeffective dose of pargyline (20 mg/kg). In contrast analogous experiments with the catecholamine precursor L-DOPA (1.0 and 10 mg/kg) did not decrease the copulatory response although motor activity (as measured by the Animax^R activity meter) was more decreased by L-DOPA 10 mg/kg than by DL-5-HTP 2.5 mg/kg. The difference in effect between L-DOPA and 5-HTP indicates that the pargyline-induced inhibition of the copulatory response was due to increased serotonin levels rather than to an increase in catechol-

amines. This is further supported by the fact that pretreatment with the serotonin synthesis inhibitor p-chlorophenylalanine prevented the inhibitory effect of pargyline on copulatory behavior in methodologically identical experiments (Malmblom and Meyerson 1971). In order to investigate the significance of increased central nervous versus peripheral monoamine levels for the copulatory behavior precursor experiments were also performed with the decarboxylase inhibitor MK486. MK486 does not pass the blood-brain barrier to any significant extent and therefore prevents the peripheral decarboxylation but not the central nervous utilization of the precursors (Porter et al. 1962; Bartholini and Pletscher 1969; Henning and Rubenson 1971). The experimental design was analogous to the pargyline + precursor experiments but MK486 was given as well. After addition of MK486 to the pargyline pretreatment the difference in effect between DL-5-HTP (2.5 mg/kg) and L-DOPA (2.5 mg/kg) became still more pronounced; whereas mount percentage was decreased after DL-5-HTP the response was increased after L-DOPA. The specificity of this difference in effect with respect to the copulatory response is demonstrated by the effects of the precursors on the overt behavior; the gross appearance and motor activity were unaffected by the dose of DL-5-HTP used while L-DOPA (in this low dose) decreased motor activity and even induced periods of immobility among the subjects. In contrast to the increased copulatory response obtained by L-DOPA 2.5 mg/kg the selective noradrenaline precursor DL-DOPS 3.0, 10 and 30 mg/kg did not facilitate the response.

Decrease of the Monoaminergic Tone (III)

A general decrease of the monoaminergic tone induced by the monoamine depletors reserpine 0.5 mg/kg and tetrabenazine 1.0 and 3.0 mg/kg decreased mount percentage. This decrease in mount percentage was not associated with a corresponding decrease in non-copulatory female-oriented activity. Like reserpine and tetraben-

zine the catecholamine synthesis inhibitor α -methyl-p-tyrosine (Spector et al. 1965) 75 and 150 mg/kg, decreased the copulatory response. α -Methyl-p-tyrosine 75 mg/kg did not decrease rotor activity or otherwise disturb the overt behavior. In contrast to the inhibitory effects obtained by α -methyl-p-tyrosine the serotonin synthesis inhibitor p-chlorophenylalanine (Koe and Weissman 1966; Jegquier et al. 1967) given 100 mg/kg for 4 consecutive days facilitated the copulatory response. The specificity of this effect is indicated by the fact that p-chlorophenylalanine in this dosage induced a pronounced decrease in rotor activity. p-Chlorophenylalanine increased mount percentage also in adrenalectomized subjects. Thus this effect was not secondary to an increased adrenal steroid hormone production.

The effects of α -methyl-p-tyrosine and p-chlorophenylalanine suggest that a decreased catecholaminergic tone inhibits and a decreased serotonergic tone facilitates the copulatory response.

α -Methyl-p-tyrosine inhibits tyrosine hydroxylase which converts tyrosine to DOPA. DOPA is then decarboxylated to dopamine. Within certain central nervous pathways dopamine is the main neurotransmitter while in other pathways noradrenaline is the main catecholaminergic transmitter (cf. Ungström 1971). Within noradrenergic neurons dopamine is converted to noradrenaline by means of dopamine- β -hydroxylase which in turn is inhibited by the compound FLA-63 (Florvall and Corrodi 1970). This drug provides a means to selectively influence the noradrenaline synthesis.

FLA-63 10 mg/kg decreased rotor activity but did not decrease mount percentage. The inhibitory effect of α -methyl-p-tyrosine on the copulatory response but lack of effect of FLA-63 suggests that an adequate dopaminergic tone rather than noradrenergic tone is essential for the copulatory response. This was further demonstrated by experiments with agents known to block catecholaminergic receptors. Pimozide which in low doses blocks dopamine but not noradrenaline receptors (Andén et al. 1970) inhibited the copulatory response at a dose level (0.25 mg/kg) which did not decrease rotor activity or otherwise disturb overt behavior. In con-

trast the noradrenaline antagonist phenoxybenzamine (Andén et al 1966 1967) did not decrease mount percentage at a dose which decreased motor activity but otherwise had little disturbing effect on overt behavior (30 mg/kg). Chlorpromazine which blocks both noradrenaline and dopamine receptors (Andén et al 1970) induced a decrease in mount percentage both after a dose that increased motor activity (10 mg/kg) and after a dose that decreased motor activity (25 mg/kg). The adrenergic β -receptor blocking agent propranolol 10 and 40 mg/kg did not affect mount percentage.

The Effect of Monoaminergic Agonists (IV)

In paper IV the effects of LSD, clonidine and apomorphine on copulatory behavior were studied. The rationale for selecting these agents was the accumulating evidence suggesting that these compounds affect monoamine receptors in a manner which would be expected by receptor stimulating agents (agonists): LSD on serotonin receptors (Andén et al 1968; Aghajanian et al 1968 1970 1972; Eliasson et al 1972; for review see Aghajanian 1972 a b) clonidine on noradrenaline receptors (Andén et al 1970a; Schmitt et al 1971; Broekkamp and van Rossum 1972) and apomorphine on dopamine receptors (Ernst 1967; Andén et al 1967; Ungarstedt et al 1969; Ungarstedt 1971a).

LSD 30 and 100 μ g/kg decreased mount percentage. At 30 μ g/kg the overt behavior was virtually unaffected and there was no effect on motor activity. Clonidine 3, 10 and 30 μ g/kg did not affect mount percentage. Apomorphine 30, 100 and 300 μ g/kg increased mount percentage. Both clonidine 30 μ g/kg and apomorphine 100 μ g/kg decreased motor activity.

CONCLUSIONS

The method developed in paper I turned out to be a useful proce-

ture for neuropharmacological studies of pre-ejaculation copulatory behavior

Mount percentage

The current data (from papers II - IV) on the influence on mount percentage of drugs affecting serotonergic noradrenergic and dopaminergic neurotransmission are summarized in table I. In this connection it must be emphasized that these changes in mount percentage have a significance only as long as the treatment regimen does not seriously impair the well-being and/or motor capabilities of the subjects. The range of doses used in this investigation fulfilled these requirements. In instances where the copulatory response was decreased this was not associated with a disturbance of overt behavior.

When the data of the present investigation are taken together it can be concluded that drugs which increase the serotonergic tone inhibit the copulatory response while drugs decreasing the serotonergic tone have the opposite effect. In contrast a drug-induced increase of the dopaminergic tone induces an increased copulatory response while a decrease of the dopaminergic tone inhibits the response. There is no support for a similar relationship for drugs affecting the noradrenergic tone.

Other copulatory parameters

Among the other copulatory parameters recorded intromission percentage followed essentially the same pattern of monoamine dependence as mount percentage. (Notable exception: propranolol decreased intromission percentage but not mount percentage.) In general an increase in mount percentage was accompanied by a decrease in mount latency and a decrease in mount percentage by an increase in mount latency. On the other hand a change in mount

latency was not necessarily associated with a change in mount percentage: clonidine decreased mount latency and DL-DOPS increased mount latency but neither drug affected mount percentage. The number of mounts/min as a rule changed in the same directions as mount percentage however the number of mounts/min was increased by FLA-63 and decreased by clonidine and DL-DOPS. The only significant changes in intramission latency was a decrease after L-DOPA and an increase after DL-5-HTP when the precursors were given after combined pargyline and MK486 pretreatment. Intramission ratio was increased after L-DOPA both with and without MK486 addition to the pargyline pretreatment and decreased by propranolol. Since the times of testing were kept very short only a very small number of subjects normally achieved ejaculation. Ejaculation percentage was however significantly increased by p-chlorophenyl-alanine and by L-DOPA given after combined pargyline and MK486 pretreatment.

Orientation of activity

Concerning the influence of the drugs tested on the orientation of activity during copulatory tests it is of particular interest that the female-oriented activity among non-counting subjects was increased by apomorphine and decreased by picroside. DL-5-HTP (after pargyline + MK486) and LSD. These findings suggest that also other components of the male rat's interaction with the female than copulatory may be under a dopaminergic/serotonergic control in a way similar to the copulatory response.

Copulatory behavior in the male and female

Copulatory behavior in the ovariectomized female rat (lordosis) is activated by estrogen followed by progesterone. There is abundant evidence that the lordotic behavior displayed by the female

TABLE I. Effect of the influence of serotonin and dopamine on mount percentage of castrated male rats maintained on ultra-low response level with respect to this parameter by means of weekly testosterone propionate treatment

AMINE	"TONE" AT RECEPTOR SITES	Mount Percentage	Drugs Tested
SEROTONIN	increased	decreased	paralyne 5-HT paralyne M404 5-HT LSD
	decreased	increased	p-chlorophenylalanine
NORADRENALINE	increased	unaffected (?)	paralyne M404 DL-COPA clonidine
	decreased	unaffected (?)	FLA-63 phenylethylamine
DOPAMINE	increased	increased	paralyne M404 L-COPA apomorphine
	decreased	decreased	n-methyl-p-tyrosine chlorpromazine pizoxide

when mounted by a male is inhibited by increased serotoninergic tone (Meyerson 1964; for review see Meyerson et al. 1973). It thus appears that serotonin has an antagonistic effect on certain components of copulatory behavior in both sexes. It is not yet clear, however, whether also catecholamines are involved in the central nervous regulation of copulatory behavior in the female and if so whether their effect is stimulatory or inhibitory.

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Lundholm, L. (Department of Pharmacology, School of Medicine Lin- 1
köping, Sweden): CYCLIC AMP AS A SECOND MESSENGER

Cyclic AMP (CA) is synthesized ($ATP \rightarrow CA + PP_i$) by the enzyme adenylyl cyclase and hydrolyzed ($CA \rightarrow 5\text{ AMP}$) by phosphodiesterase. These enzymes are present in most tissues both in animals and plants. Their activity is influenced by hormones and drugs. The tissue level of CA is regulated by the combined action of these two enzymes. The adenylyl cyclase activity is influenced by receptors associated with the enzyme. Adrenergic α - and β -effects, the action of ACTH, glucagon, acetylcholine and prostaglandins are mediated by specific receptors, which increase or reduce the adenylyl cyclase activity in the tissues. Hormones as cholecystokinin, prostaglandins and drugs as theophylline, papaverine, nitroglycerin and Ca^{++} -ions influence the CA-level through an action on the phosphodiesterase activity. A great number of metabolic reactions and physiological processes are influenced by CA. They include activation and inactivation of enzymes, release of hormones and neurotransmitters, influence on secretory processes, effects on the contraction-relaxation cycle in heart and smooth muscles and platelet function among others. CA activates a kinase which phosphorylates proteins in the presence of ATP and Mg^{++} . The nucleotide is bound to a specific CA-binding protein which in absence of CA inhibits the activity of the protein kinase. This action has been suggested to be the primary effect of CA in its regulatory function on metabolic processes. Sensitive and specific analytic methods for determination of CA have been developed by using the CA-binding protein

Osnes J B , Christoffersen T Øye I (Institute of Pharmacology University of Oslo Norway) MECHANISM OF THE INOTROPIC EFFECT OF CATECHOLAMINES AS REVEALED FROM EXPERIMENTS ON THE PERFUSED RAT HEART

It is now widely accepted that the contraction/relaxation cycle in cardiac muscle is governed by Ca^{++} ions which rhythmically become available for (and are removed from) the actomyosin-troponin complex. The amount of available Ca^{++} is supposed to limit tension development. Ca^{++} bound to the sarcoplasmic reticulum is made available for contraction by the action potential and an increase in this Ca^{++} pool may therefore increase the contractile force. Cyclic AMP increases the rate and capacity of Ca^{++} binding of cardiac sarco-tubular vesicles. This effect depends upon protein phosphorylation by a cAMP dependent protein kinase (Kirchberger M A et al J molec cell Cardiol 1972 4 673). Adrenaline and isoprenaline stimulate cardiac adenylate cyclase and increase the tissue level of cAMP. Recently it has been found that the adrenergic beta-receptor of cardiac muscle is closely linked to the catalytic site of adenylate cyclase (Lefkowitz R J Levey G S Life Sci 1972 11/II 821). These findings strongly support the hypothesis that cAMP mediates an inotropic effect of catecholamines.

We have measured cAMP levels of perfused rat hearts and the formation of ^{14}C -cAMP from ^{14}C -adenine added to the perfusate. - Inotropic response to adrenaline and isoprenaline was associated with increased cAMP. When dopamine or phenyl-ine (above $10^{-6}M$) was used to obtain an inotropic response the increase of cAMP was relatively low. Phenylephrine ($10^{-5}M$) in the presence of propranolol ($5 \times 10^{-6}M$) however caused an inotropic response without any increase in cAMP level or cAMP turnover rate. This finding supports previous observations of inotropic response to catecholamines without an increase in cAMP under certain conditions (Shanfield J et al J Pharmacol exp Ther 1969 169 315 Øye I Langslet A Adv cycl Nucleo Res 1972 1 281). We cannot exclude the possibility of a local increase in cAMP undetectable by the methods used or of merely a displacement of pre-formed cAMP. A more plausible explanation however, is that the inotropic response to catecholamines also contains a cAMP-independent component which is mediated by adrenergic alpha-receptors.

Isoprenaline increased the incorporation of ^{32}P (added to the perfusate as orthophosphate) into a TCA precipitate of heart muscle homogenate. Investigations of this phosphorylation process might serve to elucidate the difference between the biochemical events involved in the inotropic response after alpha- and beta-adrenoceptor stimulation.

Andersson R G G (Department of Pharmacology School of Medicine 3
Linköping, Sweden): CYCLIC AMP A REGULATOR SUBSTANCE IN SMOOTH
MUSCLE FUNCTION AND METABOLISM

Cyclic AMP has an important role in smooth muscle function and metabolism. After stimulation of beta-adrenoceptors by different doses of isoprenaline there was a correlation between the increase in cyclic AMP and the degree of relaxation. The relaxation was also correlated to an increase of the phosphorylase activity and a reduction of ATP content after isoprenaline and after addition of exogenous cyclic AMP. The ATP-reduction but not the increase in cyclic AMP content was dependent on the presence of Ca^{++} . The relaxing action of papaverine, nitroglycerin and theophylline was preceded by an increased cyclic AMP level and combined with an inhibition of the cyclic AMP hydrolyzing enzyme phosphodiesterase (PDE). The inhibiting action of papaverine and nitroglycerin was especially marked in the microsomal fractions which accumulated Ca^{++} . The inhibition of the PDE-activity amounted to 80-90 %.

Drugs which stimulated the PDE-activity such as imidazole in colon muscle, cholecystokinin and prostoglandin E_2 in gallbladder reduced the cyclic AMP level and contracted the smooth muscles.

The contracting action of carbacholine and acetylcholine was in rabbit colon preceded by a decreased cyclic AMP content. Secondary to the contraction there was an increase of the nucleotide content. Carbacholine reduced the cyclic AMP formation by 50 % in the microsomal fraction which released Ca^{++} by inhibition of the adenyl cyclase activity.

In the Ca^{++} -poor muscle carbacholine produced a sustained reduction of the cyclic AMP level. A sustained reduction of the cyclic AMP level after carbacholine was also observed if the muscle was pretreated with polyphloretin phosphate, a prostoglandin antagonist.

It was also shown that prostoglandin E_1 relaxed rabbit colon and increased the cyclic AMP level, the cyclic AMP increasing action of PGE_1 was in contrast to that of isoprenaline dependent on the presence of Ca^{++} . The possibility might be considered that the secondary increase in the cyclic AMP level following contraction was caused by a release of prostoglandins.

Alterations in the cyclic AMP level of smooth muscles are suggested to influence the contraction-relaxation cycle. An increase in the cyclic AMP level is suggested to relax the muscle whereas a reduction of the nucleotide level was associated with a contraction. The relaxation produced by cyclic AMP was a ATP-utilizing process. It is suggested that ATP was utilized when Ca^{++} was bound to microsomal fractions of the muscle.

4 Nilsson, K. (Department of Pharmacology, School of Medicine, Linköping, Sweden): Ca^{++} -BINDING AND RELEASE IN SMOOTH MUSCLE SARCOPLASMIC RETICULUM IN RELATION TO CYCLIC AMP

Three microsomal fractions were isolated from rabbit colon smooth muscle by differential centrifugation on a sucrose gradient. At a Ca^{++} concentration of $1.26 \times 10^{-6} \text{M}$ and at 37°C the fractions accumulated Ca^{++} in presence of ATP and Mg^{++} as shown in the table. The Ca^{++} -binding was combined with an increased ATP-hydrolysis. In absence of ATP or at 4°C the Ca^{++} -binding was much reduced. Sodiumazide (5 mM) did not influence the Ca^{++} -binding but it was reduced with 50 % by salyrgan (3 mM). The Ca^{++} -binding was influenced by the cyclic AMP concentration in the fractions (table). The binding was increased by isoprenaline which stimulated the adenylyl cyclase activity most in the 35-45 % fractions. The actions of isoprenaline was blocked by sotalol. Papaverine which reduced the phosphodiesterase activity of the fractions also stimulated the Ca^{++} -uptake. The binding was also increased by cyclic AMP itself which stimulated the ATP hydrolysis. Carbacholine and acetylcholine released Ca^{++} from the fractions and reduced their cyclic AMP content. The actions were most pronounced in the fractions isolated at 35-45 %. These actions were inhibited by atropine.

The glycerinated rabbit colon muscle was in the presence of the Ca^{++} -binding fractions contracted on addition of acetylcholine. It is suggested that drugs by influencing the cyclic AMP formation in the Ca^{++} -binding fractions stimulate the uptake or release of Ca^{++} and thereby relax or contract smooth muscle.

TABLE

Microsomal fractions of rabbit colon isolated by differential centrifugation

Per cent sucrose	35	35-45	45-55
accumulation /mg prot/10 min	65 5 ± 8 2	33 8 ± 3 0	4 8 ± 2 1
ATP hydrolysis nmol/mg prot/4 min	216 ± 29	31 7 ± 5 4	10 7 ± 3 9
cAMP-formation (pmol/mg prot/10 min)	17 8 ± 1 2	15 2 ± 1 5	20 7 ± 4 3
PDE-activity (pmol cAMP hydrolyzed/ mg prot/h)	15 5 ± 3 1	8 8 ± 2 7	0 6 ± 0 2
Action on Ca^{++} -bindings:			
cAMP (10^{-5}M) (10 min)	+20 8 ± 8 2*	+25 5 ± 6 8*	
Isoprenaline ($5 \times 10^{-7} \text{g/ml}$) (10 min)	+4 0 ± 1 3*	+31 8 ± 9 2*	
Papaverine ($5 \times 10^{-4} \text{g/ml}$) (5 min)		+12 5 ± 3 8*	
Carbacholine ($5.3 \times 10^{-7} \text{g/ml}$) (10 min)	-12 9 ± 5 0*	-30 3 ± 2 6**	

Christoffersen, T. Osnes, J. B. and Øye, I. (Institute of 5
Pharmacology University of Oslo Norway): CYCLIC AMP FOR-
MATION IN RAT LIVER: ALTERATION IN HORMONE RESPONSE WITH AGE
AND DURING TREATMENT WITH A CHEMICAL CARCINOGEN

Several cellular functions change during normal development and during malignant transformation. Alterations in the cyclic AMP system may be of particular importance in ontogenesis and oncogenesis because of the fundamental regulatory function of cyclic AMP (Robison et al. Cyclic AMP Academic Press 1971. Johnson et al. Proc nat Acad Sci USA 1971 **68** 425). We have examined the tissue levels and the formation and breakdown of cyclic AMP in rat liver from fetal life to adult age and in adult liver during treatment with the chemical carcinogen 2-acetylaminofluorene. The experiments disclosed a great similarity between the hormone response pattern of immature and preneoplastic liver in contrast to the normal adult tissue.

Adenylate cyclase activity was present one week before birth but the enzyme did not respond to adrenalin or glucagon. Two days before birth however both adrenalin and glucagon stimulated the adenylate cyclase. During the perinatal period the tissue levels of cyclic AMP, the adenylate cyclase activity and the production of cyclic AMP in slices increased rapidly reaching maximal values towards the end of the first postnatal week. This peak coincided with a maximal phosphodiesterase activity indicating an increased cyclic AMP turnover in neonatal liver. With increasing age the tissue levels and the rate of synthesis and breakdown of cyclic AMP declined. In addition the response to adrenalin and glucagon developed differently. The stimulatory effect of adrenalin on adenylate cyclase activity and cyclic AMP formation in slices gradually disappeared whereas response to glucagon persisted.

During treatment of adult rats with 2-acetylaminofluorene the response to adrenalin reappeared. This change which made the hormone stimulation pattern resemble that of immature liver occurred within one week before any histological signs of malignancy appeared.

6 Ericsson, E (Department of Pharmacology, School of Medicine, Linköping, Sweden): BETA-ADRENOCEPTOR ACTIVITY AND CYCLIC AMP METABOLISM OF RAT AORTA, VARIATIONS WITH AGE

In rat aorta the relaxing action mediated by β -adrenoceptors is reduced or absent in vessels from older animals. Since cAMP mediates this relaxation, it was investigated if the cAMP metabolism was altered. Tension was measured isometrically in aortic strips from male rats. α -adrenoceptors were blocked by phentolamine ($10 \mu\text{g/ml}$) and the preparations were contracted by serotonin ($33 \mu\text{g/ml}$).

Isoprenaline relaxed aortas from rats 1 month of age and increased their cAMP content significantly. Aortas from older animals (3 or 6 months) were relaxed very little or not at all and there was no increase of their cAMP content. Exogenous cAMP (0.1 mM) relaxed aortas from young rats markedly but the effect was minimal in aortas from older animals. Theophylline relaxed aortas from both young (1 m) and old rats (6 m) but the dose-response curve was shifted to the right in older animals. Neither basal nor NaF stimulated adenyl cyclase activity was decreased with increasing age. The phosphodiesterase activity was reduced in aortas of older rats.

The results indicate that the decreased relaxing action of isoprenaline in aortas from older rats probably depends both on a reduced sensitivity of β -adrenoceptors and a decreased sensitivity of the vessels towards the relaxing effect of cAMP.

Sørensen S C M Møller & K Møllgård (Institute of Medical Physiology Dept A & Anatomy Department A University of Copenhagen Denmark): **ALCIAN BLUE A LOW MOLECULAR WEIGHT TRACER FOR STUDIES OF THE PERMEABILITY OF INTERCELLULAR JUNCTIONS IN THE BRAIN** 7

Proteinbound dyes do not pass from blood into interstitial fluid in most areas of the brain. This has been explained by the presence of tight junctions between cerebral endothelial cells. These junctions appear in the electronmicroscope as a fusion of adjacent cell membranes in specimens fixed in glutaraldehyde and they seem to encompass the endothelial cells in their whole circumference. It is however not known if the tight junctions also represent a fusion of adjacent cell membranes in vivo. Therefore it is not known whether smaller molecules may pass through these junctions in vivo.

In an attempt to evaluate the dimensions of the tight junctions in the brain we sought a smaller tracer molecule than those which previously have been used. We have used Alcian blue which has a molecular weight of 1390. It may be seen in the light microscope but it may also be seen in the electron microscope because it forms a complex with OsO_4 .

The size of the molecule was measured on a spacefilling molecular model. In the equatorial plane the greatest distance from the center to the furthest endgroups is 12 Å. The largest distance from the equatorial plane to the furthest endgroups is 4.5 Å. In order to evaluate whether the molecule behaves as a monomer in the solutions used in our experiments we calculated Stoke's radius from viscosimetric and densitometric measurements on solutions of Alcian blue in mock cerebrospinal fluid and Locke's fluid. The calculated Stoke's radius was 8 Å indicating that Alcian blue indeed behaves as a monomer in these solutions.

The toxicity of Alcian blue was assessed by measuring O_2 -uptake in brain cortex slices from rats incubated with Alcian blue in different concentrations. When slices were incubated in a balanced medium containing 0.1% Alcian blue the concentration used for ventriculo-cisternal perfusion the respiratory rate was not affected for up to 2½ hours of incubation. Concentrations of Alcian blue above 0.5% caused a decrease in the respiratory rate.

Alcian blue does not pass from blood into brain in those areas of the brain which previously has been shown to be impermeable to proteinaceous substances. However Alcian blue does penetrate the tight junctions between ependymal cells in those areas of the brain where the ependyma has been shown to be impermeable to proteinaceous substances. Thus the tight junctions do allow passage of a substance with a Stoke's radius of 8 Å.

0 Møllgård P & S C Sørensen (Anatomy Department A & Institute of Medical Physiology Dept A University of Copenhagen Denmark): CHANGES IN CAPILLARY PERMEABILITY IN THE BRAIN STUDIED WITH ALCIAN BLUE AS A TRACER

Separating the lumen of a capillary from the brain parenchyma are two continuous layers the endothelium and the basement membrane. Normal brain capillaries are characterized by a low number of pinocytotic vesicles and by no endothelial fenestrations, reducing the passage of high molecular weight hydrophilic substances across the endothelial cells. Due to the presence of tight junctions between neighbouring endothelial cells a trans capillary intercellular passage of these same substances is also prevented.

Attempting to assess the capillary permeability to smaller molecules we used Alcian blue as a tracer substance in vivo because it is visible in the light microscope as well as the electron microscope. Alcian blue has been used widely in histochemistry as a staining and precipitating agent for heteroglycans. Basement membranes which contain polyanionic substances will be stained if the intravascular administered tracer passes through the capillary wall and furthermore a reaction product can be seen electron-microscopically in specimens treated with OsO_4 .

Alcian blue (50-150 mg/kg) was injected intravenously into anesthetized adult rats, rabbits and cats. By light microscopy Alcian blue was found extravascularly in those areas of the brain where the capillaries previously have been shown to be permeable to substances with high molecular weight (e.g. choroïd plexus, median eminence, area postrema, subfornical organ).

When the animals were breathing 25% CO_2 in O_2 for 10 min prior to and during the injection, perivascular basement membranes were stained by Alcian blue in extensive areas of the brain. This suggests that the high concentration of CO_2 increases the permeability of the endothelium.

In the electron microscopic studies Alcian blue-precipitates were found exclusively associated with the luminal part of the capillary wall in normal brain capillaries. No precipitates were seen in the pericapillary basement membranes in these areas, but in contrast Alcian blue had penetrated the fenestrated endothelial cells in areas where a high permeability is normally found and was found in the basement membranes and along the collagen fibres in the extended perivascular spaces. Alcian blue was definitely found in connexion with the endothelial fenestrations. Following exposure to CO_2 , Alcian blue-precipitates were seen in pericapillary basement membranes in extensive regions of the brain. Under these conditions the precipitates were found in the intercellular spaces between endothelial cells, suggesting that the increase in permeability is due to an opening of tight junctions.

Westergaard E and M W Brightman (Anatomy Department C, University of Copenhagen Denmark and Section on Neurocytology National Institute of Neurological Diseases and Stroke Bethesda Maryland USA): TRANSPORT OF HORSE RADISH PEROXIDASE AND FERRITIN ACROSS NORMAL CEREBRAL ARTERIOLES

Vesicular transport of intravenously injected horseradish peroxidase (HRP) occurs in the normal mouse brain across segments of arterioles with an average diameter of 15-30 μ . These vessels are pial situated primarily in cerebral and cerebellar sulci and parenchymal where they supply the ventral part of the diencephalon and neighboring areas. The transporting segments are often located at arteriolar bifurcations but involve unbranched portions of the vessels as well. Three to 30 minutes after the injection of 1 to 50 mg of HRP dialyzed against a balanced salt solution the protein reaches the perivascular basement membrane spaces and in some instances the extracellular clefts of the adjacent neuropil. As in cerebral capillaries the endothelial cells of the arterioles are linked by tight junctions impervious to HRP. Ferritin which has a larger diameter (110 A) than HRP and would therefore be even less likely to pass through the junctions was also transported across the arterioles within 3 minutes after the injection of 200 to 500 mg.

Perfusion through the cerebral ventricles of norepinephrine (100-800 μ g) for 3 minutes appeared to enhance the transport of HRP across arteriolar segments. This heightened transport was manifested by the increased density of reaction product and its more extensive spread perivascularly. Capillaries remained impermeable to HRP in all animals whether or not they were injected with amine.

10 Johansson H (Department of Neurology University of Göteborg Sweden): INCREASED CEREBROVASCULAR PERMEABILITY IN ACUTE ARTERIAL HYPERTENSION

Acute experimental hypertension induced by intravenous injection of aramine results in a blood brain barrier damage as demonstrated by spots of extravasation of Evans blue (Johansson et al Acta Neuropath 1970 16 117-124) The lesions are similar to what has been found in hypertensive crisis in experimental renal hypertension (Byrom Lancet 1954 2 201-211) The permeability change seems to be caused by the distension of the cerebral vessels as a result of the sudden increase of the intravascular pressure An abrupt pressure increase is propagated to capillaries and veins resulting in a concomitant rise of the sagittal venous pressure which indicates an abolished autoregulation (Häggendal & Johansson Acta Neurol Scand 1972 48 265-270) A stepwise pressure increase which gives the arterioles time for an autoregulatory constriction between each step does not damage the blood brain barrier even if the total blood pressure is high

An increase of the cerebral blood flow at high blood pressure levels in dogs after clamping of the aorta has been shown by Ekström-Jodal et al (Europ Neurol 1971/72 6 6-10) In the same experimental model a blood brain barrier damage can be demonstrated also after the blood flow and autoregulation have been normalized (Johansson & Linder to be published)

The concentration of amino acids- leucin and methionine- in damaged as compared to intact areas of the brain in hypertensive animals has been studied at different intervals after the acute pressure increase The amino acids have been given by intracarotid (cf Oldendorf Amer J Physiol 1971 221 1629-1639)

intravenous injection In the acute stage a passive increase the amino acids is usually found in damaged areas but preliminary results indicate that there might be a decreased transport of amino acids at a later stage

Subliminal damage of the cerebral vessels may increase their sensitivity to pressure After discrete unilateral irradiation of the brain in rabbits - the irradiation itself not being sufficient to damage the blood brain barrier - a moderate blood pressure elevation results in a marked permeability increase in the irradiated hemisphere as compared to the control hemisphere (Blomstrand Johansson & Rosengren to be published)

Eriksson E Eriksson L E Lissander B and R. Myrberg (Departments of 11
Anatomy and Physiology University of Gothenburg, Sweden):

MICROCIRCULATION IN THE TENNISIMUS MUSCLE OF THE CAT

When analyzing skeletal muscle circulation in terms of regulation mechanisms controlling flow distribution and some other local events, it has been found necessary to include the findings from the intravital microscope. We have chosen to study the vascular bed of the tenuissimus muscle in the cat. When examining the changes in blood flow at graded constrictor fibre stimulation and at administration of vasodilator drugs it became evident that the vascular anatomy of this muscle had to be further clarified. The registrations in the intravital microscope were therefore combined with the analyses of the same muscle specimens prepared for light and electron microscopy. The vascular architecture was also studied on Indian ink injected specimens prepared according to the method of Spalteholz.

The tenuissimus muscle of the cat runs from the sacro-coccygeal bones down to the crural fascia. It is a mixed skeletal muscle about two thirds of the muscle fibres being red. The average muscle fibre has a diameter of $40\mu\text{m}$. The vascular bed is composed of vessels smaller than $100\mu\text{m}$.

Morphological A-V anastomoses were practically never seen. Arterioles smaller than $10\mu\text{m}$ and venules smaller than $15\mu\text{m}$ were devoid of smooth muscle cells. No precapillary sphincters were seen. An average capillary was $101.5\mu\text{m}$ long having anastomoses to a neighbouring capillary at every $200\mu\text{m}$. At the beginning the diameter was $4.7\mu\text{m}$ in the middle $5.3\mu\text{m}$ and at the end $5.9\mu\text{m}$. The number of capillaries/muscle fibre was 0.95 and the calculated capillary surface area was $\approx 9\text{m}^2/100\text{cm}^3$ of muscle tissue.

No vasomotion was seen. The average red cell velocity in capillaries was $\approx 5\text{mm/s}$ with a range from 0 up to 15mm/s . The blood cells especially the leucocytes seemed to influence the flow velocity in a capillary where the flow rate decreased with an increased hematocrit and vice versa. All vessels furnished with smooth muscle cells showed diameter changes on sympathetic stimulation and administration of vasodilator drugs the most marked ones occurring in arterioles of $15\text{--}20\mu\text{m}$.

12 Nicolaysen, S (Institute of Physiology University of Oslo Norway): TRANSVASCULAR FLUID BALANCE AND PROTEIN PERMEABILITY IN THE MICROVESSELS OF THE LUNG

Some recent investigations have added to the understanding of transvascular fluid balance and protein permeability in the microvessels of the lungs. It is generally accepted that Starling's equation for transvascular fluid exchange also applies to the lung. The observations of Guyton and Lindsey (Circulat Res 1959 7 649) seemed difficult to explain on that basis: They observed no increases in extravascular lung water content upon increases in left atrial pressure up to 25 mm Hg for up to 2 hrs in intact dogs. However Staub and coll (e.g. Staub N C et al Microvasc Res 1972 4 331; Vaughan T R jr et al Clin Res 1972 20 583) have been able to supply data that probably explain the puzzle. They chronically cannulated lung lymphatics in sheep. In the unanaesthetized animals they found a nearly linear correlation between microvascular pressure and lung lymph flow with lymph flowing also at normal microvascular pressures. They also confirmed Guyton and Lindsey's observations on lung water content. No increase in extravascular lung water on increases in microvascular pressure up to 25 mm Hg for at least 2 hrs. As lymph flow increased upon increases in microvascular pressure the concentration of protein in the lung lymph decreased. Thus two mechanisms will tend to keep the lung dry: a) a lymph flow that increases on demand and b) a lymph protein concentration that varies with lymph flow. This last thing probably indicates that the interstitial colloid osmotic pressure varies in such a way that an increase in microvascular pressure will be partially offset by a decrease in interstitial colloid osmotic pressure. This last-mentioned mechanism has previously been proposed by Lunde and Waaler (J Physiol 1969 205 1) on the basis of experiments on isolated lungs. Staub and coll in their experiments also found a rather rapid blood to lymph equilibration of tracer albumin in the sheep with a half time of about 2-2½ hrs. Similar observations were done by Boyd et al (J Physiol 1969 201 567). These data point to a fairly high permeability of the microvessels to proteins. On the other hand electronmicroscopical studies with protein tracers failed to show detectable transfer of the protein to the interstitium within 30 min after the intravenous injection (Pietra G G et al Science 1969 166 1643; Schneeberger E E and Karnovsky M J J Cell Biol 1971 49 319; Nicolaysen and Staub (in prep) have qualitatively determined the time course of the vascular-extravascular equilibration of albumin in mice lungs using the bright red fluorescence of Evans blue labelled serum albumin. Equilibration appeared to be reached within 3-5 hrs of the i.v. injection of the tracer substance. Interstitial tracer was observed already 20 min after the injection. Also autoradiographic studies with ¹²⁵I albumin suggested advanced equilibration at ½ hrs after the injection. This study thus lends support to the notion that the permeability of the microvessels in the lungs to proteins is not negligible.

Groth T and M A Lassen (Uppsala University Data Center, Uppsala Sweden and Department of Clinical Physiology, Bispebjerg Hospital Copenhagen Denmark):

DOES THE GLOMERULAR MEMBRANE OPERATE AS A MILLI-PORE FILTER?

In a previous study of the human glomerular membrane (Arthurson U Groth T and Grotte G Clin. Sci 1971 40:137) it was shown that a strict non-linear analysis of dextran sieving data in terms of a two-pore model leads to a surprisingly low value of the mean transglomerular pressure difference (less than 1 mmHg). The corresponding value of the effective pore area was found to largely exceed the total capillary area as estimated by histological techniques (Vimtrup, B J Amer. J. Anat., 1928 41:123).

The calculations yield an estimate of the surface area per unit path length $A/\Delta x$. The discrepancy mentioned was caused by inserting the entire thickness of the glomerular basement membrane (about 3000 Å) in the $A/\Delta x$ expression.

In the present communication the discrepancy is discussed. If the critically narrow part of the channels of the glomerular filter is of the order 10-50 Å and not 3000 Å then values for the effective pore area can be obtained that are compatible with anatomical estimates. The remaining part of the glomerular basement membrane must then have considerably wider dimensions of its presumably mesh-like system of water filled channels through which the ultrafiltrate passes. This solution gives quantitative support to the hypothesis that the glomerular membrane should operate like a millipore filter. Such filters have a critically narrow part of its channels occupying only a very small fraction of the thickness of the filter. The critically narrow part must face the solution from which ultrafiltrate is formed: the filter simply cannot function in the opposite direction because the impermeant solute will clog it up if one tries to reverse the filter.

Thus we are led to suggest that also in the glomerulus the true filter that practically excludes colloids from sieving into the ultrafiltrate, is placed on the blood side (in the fenestrae). The remaining part of the filter essentially constitutes a mechanical support through which the ultrafiltrate passes without further being modified. In this hypothesis the electromicroscopical demonstration of some large tracer molecules penetrating fairly deep into the glomerular membrane is not indicative of the typical mode of operation but rather of imperfections in the filter occasionally letting larger molecules penetrate deeper than the critical surface layer.

- 14 Bergström Sune (Karolinska Institutet 104 01 Stockholm);

THE PROSTAGLANDINS AND THEIR METABOLISM

During recent years the involvement of the prostaglandins in the regulation in many physiological processes has been studied

Natural prostaglandins and some new analogues show great promise as drugs in several fields of medicine

A short review will be presented on recent developments that might be of interest as a background for the other contributions to the symposium

- 1 Kjnqård E and C Larsson (Department of Pharmacology Karolinska Institutet S-104 01 Stockholm 60 Sweden)
PROSTAGLANDIN MEDIATED HYPOTENSIVE EFFECTS OF ARACHIDONIC ACID IN THE RABBIT

The effect of arachidonic acid (C 20:4) a prostaglandin precursor was studied on the blood pressure and the urinary output of prostaglandins (PG) in rabbits C 20:4 induced a dose dependent decrease in the blood pressure Intrarterial infusion of 15-25ug/kg/min reduced the blood pressure by 10-21% (S D) 30-45ug/kg/min by 21-31 50-90ug/kg/min by 34-61 and 95-150ug/kg/min by 61-91 The urinary PG identified by bioassay in combination with chromatography and 15-hydroxyprostaglandin dehydrogenase inactivation increased by 70% (n=3) Following indomethacin (10mg/kg) or S 8 11 14-eicosatetrayonic acid (10mg/kg) two structurally different PG synthesis inhibitors the blood pressure increased by 20-11 (p < 0.001) and subsequent infusions of C 20:4 (50-200ug/kg/min) failed to elicit any hypotensive effects or an increase in the urinary PG The results suggest a role for endogenous PG in regulating the blood pressure of the rabbit

Kjasson R H Bygdeman and P Eneroth Dept Physiology Karolinska 16
Institutet and Dept Obstetrics & Gynecology Karolinska Hospital Stockholm
Sweden); EFFECTS OF HORMONES ON PROSTAGLANDINS IN HUMAN SEMEN

The aim was to investigate effects of exogenous hormones on the content of various prostaglandins (PG) in human seminal plasma. Patients referred due to infertility were selected for the study only if there were medical reasons for endocrine treatment. A few volunteer medical students were included in some experiments. Gonadotropins were given as i.m. injections of Humegon (75-150 IU HMG 3 or 6 times a week) and/or Pregnyl (500-1500 IU HCG at the same time intervals) for 3 to 6 months. Androgens were given either orally (methyltestosterone 10 to 30 mg a day for 10 to 30 days) or i.m. in the form of longacting preparations (Triolandren/CIBA or Testoviron-Depot/Schering AG) every third week.

The seminal plasma was analysed chemically for PGE, PGA + PGB and 19OH-PGA + 19OH-PGB; in most cases also acid phosphatase activity, zinc and fructose.

In most patients the PG content was not significantly changed by administration of the various hormones. In some patients the seminal PG content decreased. Nor were the other chemical parameters - as a rule - altered.

The results are at variance with those reported by Sturd (Arnsperg-Forsch 1971 21 986) who used bio-assay on isolated guinea-pig ileum and report that gonadotropin treatment caused a doubling of the PG-activity in human seminal plasma.

Factors controlling the occurrence of different PG in human semen warrant further studies.

Hedqvist, P. (Department of Physiology Karolinska Institute Stock 17
holm Sweden); ASPECTS ON PROSTAGLANDIN MEDIATED CONTROL
OF ADRENERGIC TRANSMITTER RELEASE

Various mechanisms have been envisaged for the control of transmitter homeostasis in adrenergic nerves. This paper describes the influence on noradrenaline (NA) release from adrenergic nerves by prostaglandins (PGs) and agents affecting the α receptors.

Isolated guinea pig vasa deferentia preincubated with ^3H NA were superfused with Tyrod solution and were transmurally stimulated (NS) with square wave pulses. NS caused the release of NA and of small amounts of PGE like material. Very low doses of PGE₁ and PGE₂ caused a reversible and dose dependent inhibition of NA overflow in response to NS. The inhibition varied inversely with the stimulation frequency and with the calcium concentration in the external medium. Inhibition of local PG formation increased NA overflow in response to NS. α receptor blocking agents increased and compounds with α receptor stimulating properties decreased NA overflow in response to NS even after proper pharmacological blockade of NA uptake mechanisms and of local PG formation.

It is suggested that NA release induced by nerve action potentials activates presumed presynaptic α receptors and triggers PG formation and that both the α receptor and the PG are capable to modulate further release of NA from the neuron.

18 Janson, P O and K. Åhrén (Department of Physiology and Department of Obstetrics and Gynecology University of Göteborg Sweden): EFFECTS OF PROSTAGLANDINS (F2 α) ON OVARIAN BLOOD FLOW

It has been proposed that prostaglandin F2 α exerts its luteolytic action by a vascular mechanism, leading to relative ovarian ischemia. In the present study anesthetized pseudopregnant rabbits on days 5-9 of luteal phase received PGF2 α i.v. at a dosage of 50 μ g/kg. Before, during and for 30 min after PG administration heart rate and systemic arterial pressure were monitored.

In one group of animals the blood flow of the ovarian vein was measured during the above mentioned time period. In another group the ovarian blood flow was measured by means of 188Ytterbium and 46Scandium-labelled 35 \pm 5 μ microspheres before and at different time periods after PG injection.

PGF2 α i.v. caused a marked arterial hypotonia and bradycardia for 20-30 min. These effects were then normalized. This response to PG could be modified by atropine i.v. or by vagotomy.

There were acute reductions in blood flow of the ovarian vein, the whole ovary and the corpus luteum during the phase of arterial hypotonia. The blood flow values returned to their original levels at the same time as the systemic pressure was normalized.

In these studies of the rabbit ovarian blood flow no specific effect of i.v. PGF2 α could be observed.

7 C and E Ånggård (Department of Pharmacology Karolinska Institutet S 104 01 Stockholm 60 Sweden) INCREASED JUXTAMEDULLARY BLOOD FLOW FOLLOWING STIMULATION OF INTRARENAL PROSTAGLANDIN BIOSYNTHESIS

The renal blood flow distribution was studied before and during stimulation of intrarenal prostaglandin (PG) formation by infusion of the precursor arachidonic acid (C20:4).

C20:4 was infused into the aorta above the origin of the renal arteries. The regional distribution of renal cortical blood flow was determined by injecting 51Cr and 141Ce labelled microspheres (15 \pm 5 μ) into the left ventricle before and during arachidonate infusion respectively. The count rates of 141Ce to 51Cr were determined in consecutive slices from cortex to inner medulla. Urine was collected from the ureters and analyzed for prostaglandins using bioassay in combination with specific enzymatic inactivation and silicic acid and gas liquid chromatography.

Non-hypotensive doses of C20:4 increased the blood flow in the juxtamedullary cortex by 25 \pm 5% and caused an increase of 50 \pm 16% of the output of urinary PG, mostly PGE2. Indomethacin decreased the juxtamedullary blood flow by 37 \pm 5% and the urinary PG by 42 \pm 12% and antagonized the effects of C20:4 on the renal blood flow and urinary PG. Our results provide evidence for a role of intrarenal PGE2 as a mediator of autoregulation of renal blood flow.

Søndergaard, J (Department of Dermatology Rigshospital Uni-20
versity of Copenhagen Denmark): CUTANEOUS INFLAMMATION IN-
DUCED BY PROSTAGLANDIN E₁

The effect of prostaglandin E₁ (PGE₁) on human cutaneous vasculature skin histamine and dermal connective tissue has been studied. A single intradermal injection of PGE₁ caused a persistent dusky erythema and a well-defined wheal. The relationship of the magnitude of the erythema and the wheal to dosage of PGE₁ was established in 16 subjects. Intradermal injection of 1 µg PGE₁ reduced skin histamine in 10 subjects by approximately 24%. Part of the cutaneous reaction to PGE₁ was probably due to the observed endogenous histamine release since the wheal but not the erythema, was reduced by pretreatment with antihistamine. Seven injections of 5 µg PGE₁ given intradermally at intervals of 8 hours resulted in a cutaneous reaction clinically characterized by redness, edema and tenderness. Microscopic examination of the PGE₁-treated skin revealed a moderate mononuclear-cell infiltrate located around the dermal vessels and epidermal appendages. Biochemical analyses of PGE₁-treated skin biopsies revealed an increase in the concentration as well as in the total amount of hyaluronic acid as compared with that in saline-treated control biopsies.

The results indicate that PGE₁ may initiate regenerative processes in human dermal connective tissue.

This work was supported by a grant from the Danish Medical Research Foundation.

Strandberg, K (Department of Pharmacology Karolinska Institutet 104 01 Stockholm 60 Sweden): BRONCHOCONSTRICTOR ACTIVITIES OF HISTAMINE, PROSTAGLANDIN F_{2α} AND SLOW REACTING SUBSTANCE

Bronchoconstricting agents, such as histamine, prostaglandin F_{2α} (PGF_{2α}) and slow reacting substance (SRS) appear in anaphylactic reaction in guinea-pig lung tissue (Piper P J and J R Vane Nature 1969 223 29). In the present investigation the effects of these substances on the airway pressure in anaesthetized guinea-pigs were compared using the Konzett-Rössler technique.

Intravenous injection or aerosol administration of the constricting agents caused a dose-dependent increase in airway pressure. The onset and duration of action were longer for PGF_{2α} and SRS than for histamine. The systemic blood pressure was usually increased by i.v. injections of PGF_{2α} and SRS whereas when influenced it was lowered on aerosol administration of all the substances. The approximate ratio between the dose needed to produce the same peak effect (airway pressure) on i.v. injection as on aerosol administration was: 1 (0.1-2.5) for histamine, 28 (5-100) for PGF_{2α}, and 3 (0.3-10) for SRS.

It is concluded that histamine, PGF_{2α} and SRS can produce an increase in the airway pressure in the guinea-pig and that the magnitude of this effect is dependent on the administration route chosen.

- 22 Svanborg N, Hamberg, M, Hedqvist P (Med. Dept. Nacka Hospital and Depts Med., Chem. and Physiol. Karolinska Institute Stockholm Sweden) ASPECTS ON PROSTAGLANDIN ACTION IN ASTHMA. Prostaglandins (PGs) are normal constituents of lung tissue and are released from isolated guinea pig and rat lung by various stimuli. This paper describes the effect of inhaled PGFs on airway conductance (SG_A) and of inhaled specific allergen on urinary excretion of PG in patients with allergic asthma.
- PGs and allergens were administered as aerosols and SG_A was measured in a whole body plethysmograph. 5α and 7α dihydroxy 11-keto tetra α prostano 1,16-dioic acid, the major urinary metabolite of $PGF_{2\alpha}$ and $PGF_{2\beta}$ was determined by multiple ion analysis.
- $PGF_{2\alpha}$ reduced SG_A in both healthy subjects and asthmatic patients; the latter being approximately 10000 times more sensitive to the compound. $PGF_{2\beta}$ (the stereoisomer of $PGF_{2\alpha}$) did not elicit bronchoconstriction but rather a modest increase in SG_A . Moderate allergen provoked asthmatic attacks did not significantly alter urinary excretion of the PGF metabolite while an unexpectedly severe and longlasting attack almost doubled the excretion. Premedication with indomethacin almost completely abolished the excretion of the PGF metabolite in urine and meanwhile markedly reduced the sensitivity to allergen provocation.
- Hyperreactivity to $PGF_{2\alpha}$ possibly coupled with overproduction of the compound is suggested to be of significance in the pathogenesis of allergic asthma.

- 23 Wennmalm Å and M. Junsted (Fysiologiska Inst. I Karolinska Inst. Stockholm Sweden) ENDOGENOUS PROSTAGLANDIN MEDIATED INHIBITION OF PARASYMPATHETIC NEUROTRANSMISSION IN THE RABBIT HEART ?
- It has earlier been shown that the release of noradrenaline from sympathetic nerve endings is restricted by an endogenous prostaglandin (PG) mediated control mechanism and that the release of acetylcholine (ACh) from parasympathetic nerves is inhibited by exogenously administered PGE₁ (Wennmalm Å. Acta physiol scand 1971 83 Suppl 365). A prerequisite for the endogenous control of sympathetic transmitter release is liberation of PG in response to nerve stimulation. In the present paper we wish to report release of PG following parasympathetic nerve stimulation or ACh infusion.
- Rabbit hearts with intact vagal nerve supply were perfused with Tyrode solution according to the Langendorff technique. Right and left vagal nerves were stimulated supramaximally at a frequency of 5 Hz for 10-12 min. Perfusate from the heart was collected both at rest and during nerve stimulation. In some hearts ACh was infused (28-96 µg during 7-12 min). Lipids in the perfusate were extracted with ethyl acetate. They were identified using T.L.C. and quantified on a superfused rat stomach. At rest a small outflow of PG (4.5 ng/min) from the heart was observed. During vagal nerve stimulation this outflow was increased to 11.5 ng/min (39 pg/imp). Infusion of ACh also facilitated the liberation of PG to 13.5 ng/min (2 ng/µg ACh infused). Atropin 1 mg/l neither influenced the nerve stimulation nor the ACh infusion induced outflow of PG. Since exogenous PG can inhibit ACh liberation in this organ, the present experiments suggest that parasympathetic neurotransmitter release in analogy with sympathetic is restricted by endogenously synthesized prostaglandins.

Jacobsen S (Institute of Pharmacology B University of Oslo 24
Norway): METHODS AND EXPERIMENTAL MODELS IN PROTEIN BINDING
STUDIES

The interactions of organic compounds with macromolecules are of general interest in biology. However the experimental results concerning binding parameters are not always directly relevant to in vivo conditions and results from different laboratories are not comparable due to the different methods used to evaluate binding parameters.

The aim of this paper is mainly to give a survey of the different methods used to determine protein binding and to discuss the problems and the pitfalls in the evaluation of binding characteristics as the number of binding sites and the strength of binding. The influence of inorganic ions and endogenous substances on the binding will be demonstrated.

The binding characteristics of different drugs to serum albumin are presented to elucidate the general topics of the paper.

Nilssen O G (Institute of Pharmacology B University of Oslo 25
Norway): THE BINDING OF QUINIDINE TO SUBFRACTIONATED HUMAN
SERUM PROTEINS

It has been shown previously that many drugs are bound to serum albumin to a large extent. However the total binding of many drugs in the human serum are not explained by binding to albumin only. Human serum has been fractionated by gel-filtration and ultracentrifugation. The binding of quinidine to the proteins was determined by equilibrium dialysis.

Our studies demonstrate that albumin is only one of the important drugbinding proteins in the human serum. As for the weak base quinidine it seems that of the total quinidine bound in normal human serum 50% is bound to albumin and the other serum fractions of higher molecular weight account for the remaining 50%. The serum lipids other than FFA seem to interact with the binding of quinidine to serum proteins.

It is concluded that serum albumin, the high molecular fractions and the lipids must be considered to evaluate the binding of drugs in serum.

- 26 Fremstad D (Institute of Pharmacology University of Oslo Norway): INFLUENCE OF PLASMA AND TISSUE BINDING ON THE DISTRIBUTION OF QUINIDINE AND PHENYTOIN IN THE RAT

The plasma protein binding of the basic drug Quinidine and the acidic drug Phenytoin was increased and reduced respectively in rats with acute uremia induced by ligation of the ureters. The binding of the drugs in plasma and tissue homogenates from normal and uremic rats was determined by equilibrium dialysis. The in vivo distribution of the drugs was studied at an apparent equilibrium after i.v. injections in normal and uremic rats.

The distribution of the drugs was expressed as the ratios R_t = total concentration in tissue/total concentration in plasma and R_f = total concentration in tissue/concentration of unbound drug in plasma. R_t was different and R_f the same in normal and uremic rats. An estimate of the drug distribution to the heart based on the binding parameters obtained in homogenates was in accordance with the distribution in vivo for Phenytoin but not for Quinidine. The last discrepancy can be eliminated if one assumes an intracellular pH of 7.3 or cell membranes permeable to both ionized and unionized Quinidine.

The findings indicate that the plasma protein and the tissue binding of the drugs determine distribution to the heart. The observed changes in tissue distribution in uremic rats are mainly due to changes in plasma proteins.

- 27 Odar-Cederlöf I, and Borgö O (Department of Internal Medicine Karolinska Hospital and Department of Clinical Pharmacology, Huddinge University Hospital Stockholm, Sweden): PLASMA PROTEIN BINDING AND PHARMACOKINETICS OF DIPHENYHYDANTOIN IN UREMIC PATIENTS

Plasma protein binding of diphenylhydantoin (DPH) was lower in uremic patients than in normal persons. This seemed to depend mainly on a decreased affinity constant of albumin for DPH in uremia. Pharmacokinetic investigation using single intravenous doses showed that in uremic patients whose unbound fraction of DPH was higher total plasma DPH concentrations were lower and values of the apparent volume of distribution V_d higher than in normal subjects. Plasma concentrations and values of V_d for unbound drug were similar in the two groups. Thus equivalent doses give rise to equivalent unbound concentrations of drug and probably to the same effects but total plasma concentrations differ in the two groups causing a different total plasma concentration - effect ratio in uremic patients.

Borgå, O (Department of Clinical Pharmacology at Karolinska Institutet, Huddinge University Hospital S-141 86 Huddinge, Sweden): PHARMACOLOGICAL CONSEQUENCES OF PLASMA PROTEIN BINDING 28

The importance of plasma protein binding of a drug for its pharmacological effects is often overemphasized. One reason for this might be that results obtained *in vitro* have been directly extrapolated to the *in vivo* situation, without paying attention to the actual pharmacokinetic parameters of the drug.

The apparent volume of distribution, V_d , of the drug seems to be a parameter which is of great value in predicting the consequences of a change in the plasma protein binding caused by e.g. a displacement interaction. Thus a potentiation of the pharmacological effect is theoretically possible only for drugs with a low V_d (0-10 l/kg). For a drug with a high V_d , the displacement interaction will lead to decreased plasma concentrations without significantly increased unbound drug levels in the body. At an equilibrium state the decreased plasma protein binding for such a drug might not significantly effect metabolic or renal elimination rates or pharmacological response.

Dahl, S G (Institute of Pharmacology University of Oslo): RELEVANCE OF PROTEIN BINDING FOR PHARMACOKINETIC MODELS 29

In clinical pharmacology pharmacokinetic models have proved to be valuable tools for describing the time-course of drug concentrations in blood and urine after administration of single and multiple doses.

In a pharmacokinetic model the absorption, distribution and elimination of drugs are described in simple mathematical terms and the reliability of the result depends on how accurately these processes are described. It is well known that the binding of drugs to plasma and tissue proteins may influence their pharmacokinetics as for example in the interaction between warfarin and phenylbutazone (O Røilly R A and Levy, G J Pharm Sci 1970 59, 1258).

Some pharmacokinetic models that take into account the binding of drugs to plasma proteins will be presented. Unfortunately their usefulness is reduced by complicated mathematical treatment.

Practically simpler models which do not account for drug protein binding may have to be used. The assumptions involved in these models will be discussed and methods for testing their validity will be given.

The original and classical evidence for the existence of osmoreceptors in the hypothalamic region of the brain and for a central osmometric control of water balance was obtained by studying the effects of alterations in the solute composition of the blood plasma (Verney E B Proc roy Soc B 1947 135 25). Later studies of central sodium-angiotensin interaction performed with the stimuli applied from the inside of the blood-brain barrier (BBB) suggest that a possible alternative to hypothalamic osmoreceptors may be receptors in the close vicinity of the third cerebral ventricle which respond to changes in the Na^+ concentration of the cerebrospinal fluid (CSF) (Anderson B Amer Scientist 1971 59:408).

Recent studies of osmotic and other stimuli applied both from the outside and from the inside of the BBB in the conscious goat seem to contradict the osmoreceptor theory and support the idea of a periventricular Na^+ sensitive receptor system of importance in the control of water and salt balances. A rise in the carotid blood osmolality obtained by infusions of hypertonic NaCl and fructose acts as a much more potent stimulus to thirst and release of antidiuretic hormone (ADH) than the equivalent rise elicited by glucose, galactose, glycerol and urea. A rather effective BBB exists for all these substances (Crane C Acta physiol scand 1965 64 407; Yudelevich D L and de Rose N Amer J Physiol 1971 220 841) and their intracarotid application in hypertonic solutions ought to cause approximately the same degree of brain dehydration. Therefore the observed differences in dipsogenic and antidiuretic effects appear incompatible with the idea that osmoreceptors are located inside the BBB. Further evidence against this idea is the observation that infusions of hypertonic fructose and sucrose into the cerebral ventricular system neither elicit thirst nor release of ADH. In contrast to corresponding infusions of hypertonic NaCl.

An alternative would be that cerebral osmoreceptors of importance in fluid balance were located outside the BBB or in a region of the brain which lacks an effective barrier of this kind. However slow infusions of iso- or hypertonic solutions of sucrose or monosaccharides into the lateral cerebral ventricle repress the dipsogenic, antidiuretic and natriuretic responses to intracarotid infusions of hypertonic NaCl. This would hardly be the case if the intracarotid infusions of hypertonic NaCl acted via receptors located outside the BBB. Rather this repressive effect of non-electrolytes infused into the ventricular system seems to be due to a dilution of the CSF Na^+ concentration since no repression of the effects of an intracarotid NaCl infusion is obtained when the non-electrolytes are administered into the ventricle dissolved in isotonic saline.

Adding small amounts of angiotensin II to intracarotid infusions of hypertonic NaCl and fructose potentiates the ADH releasing and dipsogenic responses. If this potentiation reflects a central sodium-angiotensin interaction it indicates that the intracarotid infusions of hypertonic fructose act dipsogenic and antidiuretic indirectly by inducing a rise in the CSF Na^+ concentration. The eventual demonstration that intracarotid infusions of hypertonic fructose (in contrast to equivalent infusions of other monosaccharides) raise the CSF Na^+ concentration would provide additional support for the idea that a periventricular Na^+ sensitive receptor system plays an important role in the central control of body fluid homeostasis. Furthermore it would indicate that the blood-liquor barrier is of greater importance for the central control of fluid balance than is the BBB.

The antidiuretic hormone under normal circumstances is the principal factor in the regulation of renal water excretion. This hormone therefore participates in the maintenance of both extracellular volume and strength. The stimuli which determine the secretion rate of ADH are still incompletely characterized although the total osmotic activity of the extracellular fluid (TOA) and the (thoracic venous) blood volume frequently are described as the dominating factors corresponding to the dual nature of the effect of ADH. Blood volume. Initiated with the findings of Leaf & Mamby (1952) evidence has now accumulated to a degree that makes the significance of this stimulus appear unambiguous. The regulatory mechanism depends on stretch receptors located in the left atrium in connection with intact vagus nerves making the fullness of the low pressure system influence the ADH secretion rate. The role of high-pressure baroreceptors in the normal regulatory range seems insignificant but these receptors (carotid sinus aorta) have been found to affect ADH release under certain conditions. Osmotic activity: The experiments of Verney (1947) might suggest that TOA influences the release rate of ADH, but the results are only convincing with respect to unphysiologically high stimulus intensities. Later work on the subject is scanty and hardly conclusive so the theory of osmoreception is still open to criticism.

In order to evaluate further the role of the so-called osmoreceptors a technique has been developed which is based on Verney's principle (intracarotid infusions of hypertonic solutions into hydrated dogs) modified in several ways: a) light chloralose anesthesia was used b) hydration fluid was a hypotonic solution given intravenously c) hydration was maintained (3-4 % BW) by means of a weighing operating table controlling an infusion pump (Jørgensen & Bie (1972)), d) experiments were also performed on dogs in which selective cardiac vagotomy had been made previously in order to avoid volume receptor input on the neurohypophysis. The dogs respond properly to the U S P reference standard (80 μ U ADH/kg BW bolus injection) and therefore offer an opportunity of repetition and evaluation of the results of Verney.

References:

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Leaf A & Mamby A R : J Clin Invest 31(1952)80
Verney E B : Proc Roy Soc Ser B : 135(1947)225

32 Bojesen E (Inst of Experimental Hormone Research University of Copenhagen Denmark): INTRARENAL CONTROLLING SYSTEMS THEORIES AND FACTS

Research into intrarenal control systems specifically involving tubular processes will undoubtedly be intensified when the implications of the load independency of proximal reabsorption and nearly complete filtration equilibrium across the glomerular membrane become fully realized. As pointed out almost 20 years ago such phenomena emphasize that the rate of formation and composition of the urine is the resultant of two kinds of properties of the entire nephron: i.e. reabsorptive capacities and fluid flow characteristics both of which are greatly changing along the nephron. By simply considering finality or selection one can predict that such a complicated arrangement must be equipped with servo control functions in order to be reliable since in their absence any setting of one of the two kinds of nephron properties will greatly influence the result of any change in the other.

Most work on intrarenal control mechanisms have until recently been concerned with the juxtaglomerular apparatus (JGA) and the renin system with tubules and/or vessels as targets in a short feedback loop. Our observations on interstitial cells (IC) of the medulla suggest however the existence of a second regulatory system. Morphologically this extensive cellular system is equipped as secretory cells with lipid droplets as the characteristic constituent. The main component of the droplets is triglycerides which in rodents has a high content of polyunsaturated fatty acids, a major fraction of which is adrenic acid ($22:4 \pm 6$). This acid is produced in vitro by chain elongation of arachidonic acid ($20:4 \pm 6$) of local origin. The number of lipid droplets per cell at the tip of the papilla increases rapidly after water or saline loading. Thus far no product of known biological activity has been identified although the cells have been assumed to produce prostaglandins. More knowledge about the prostaglandins in general and also their relationship to the kidney now makes it unlikely that the cells are merely prostaglandin producing. Thus our observation that the adrenic acid is produced and incorporated into triglycerides of the droplet and phosphatides indicates a trapping mechanism for the prostaglandin precursor arachidonic acid.

Three results in our recent studies on the sterologenesi of papillary tissue are consistent with the idea that a member of this lipid class is important perhaps locally. The rate of sterologenesi is high, sterols are not esterified locally and desmosterol is apparently an end product accumulated in the tissue.

Unfortunately it is still premature to postulate any particular role of the IC system but we consider it entirely possible that there is overlapping and/or cooperation with the JGA system.

The rise in sodium excretion after salt intake is associated with expansion of the extra-cellular volume. Natriuresis is partly due to a rise in glomerular filtration rate (GFR) and partly to inhibition of proximal tubular reabsorption. To distinguish between changes in passive and active energy-requiring tubular transport we have in addition to measurements of renal oxygen consumption used the heat accumulation technique to estimate variations in metabolic rate in cortex and outer medulla. Changes in GFR in dogs and corresponding changes in proximal tubular reabsorption (glomerulotubular balance) were associated with only small changes in renal energy requirement (Sejersted et al. *Am. J. Physiol.* 220: 1488-1493 1971. Lie et al. *Am. J. Physiol.* in press). In contrast a rise in sodium reabsorption (and free water generation) in the ascending limb of Henle's loop during increased delivery from the proximal tubules is associated with corresponding increments in metabolic rate in the outer medulla. Thus saline loading increases GFR and inhibits proximal tubular net reabsorption without altering active transport. The increased delivery to the distal nephron is partly compensated for by a rise in active reabsorption in the ascending limb of Henle's loop presumably mainly by raising Na-K-ATPase activity. This effect is blocked by ethacrynic acid and ouabain.

After inhibition of sodium reabsorption with ethacrynic acid the distal nephron is expanded and the hydrodynamic resistance of the nephron reduced towards minimum, under these conditions a change in urine flow during saline and mannitol infusion or ureteral obstruction was found to vary in proportion to tubular driving force (P) the difference between intrarenal pressure in the cortex and ureteral pressure (Kiil et al. *Am. J. Physiol.* 223: 1263-1270 1972). Further examinations on unblocked kidneys (Osvik et al.) showed that tubular dilatation on account of a rise in tubular transmural pressure was the main tubular hydrodynamic event during saline infusion; the rise in P could only account for a doubling of urine flow. In other studies (Bader et al.) the hemodynamic mechanisms contributing to passive changes in proximal reabsorption were investigated by injecting saline at high and control perfusion pressure in dogs. At renal perfusion pressure of 170 mm Hg saline injection abruptly increased renal blood flow intrarenal pressure sodium excretion and urine flow whereas the responses were smaller at 110 mm Hg and barely perceptible at 80 mm Hg. GFR was essentially constant at perfusion pressures exceeding 110 mm Hg and the highest natriuretic effect at high perfusion pressure therefore a consequence of tubular inhibition. The exaggerated natriuretic response on acute elevation of renal perfusion pressure can be explained in terms of the autoregulation hypothesis of salt excretion (Kiil et al. *Acta physiol scand* 76: 24-39 1969. Kiil *Scand J clin Lab Invest* 25: 113-117 1970).

34 Öberg, B Dept of Physiology University of Göteborg Sweden: THE INFLUENCE OF CARDIOVASCULAR RECEPTORS ON EXTRACELLULAR FLUID DISTRIBUTION AND RENAL WATER AND SALT EXCRETION

Cardiovascular receptors, sensing the fullness and the prevailing performance of the cardiovascular system have been assigned important roles in the control of extracellular fluid volume (e.g. Gauer Henry and Behn 1970). This volume regulation includes both the internal distribution of available extracellular fluid between intra- and extravascular compartments and the external fluid exchange with the environment primarily by affecting the renal handling of salt and water.

Redistribution of fluid between intra- and extravascular compartments by way of the capillary filtration absorption process is effected by reflex resetting of the pre/postcapillary resistance ratio leading to appropriate shifts in the capillary hydrostatic pressure. Such reflex adjustments of the resistance ratio result primarily from removal of the inhibitory restraint from the arterial baroreceptors and are most pronounced in the skeletal muscles which due to their large mass constitute the most important extravascular fluid depot in the organism (Öberg 1964). Through this mechanism the plasma volume can be maintained at the expense of the interstitial fluid content.

Assuming that a quantitative relationship normally exists between the fluid contents of the intravascular and the total extracellular compartments a disturbance in the total extracellular fluid volume should influence cardiovascular receptors located mainly in the low pressure part of the circulatory system e.g. in the heart. The efferent pathways of volume regulating reflexes from cardiac receptors include both hormonal mechanisms e.g. the release of ADH and neuronal mechanisms e.g. the renal vasoconstrictor fibres with consequent reflex influences on renal hemodynamics, renin release and on tubular function. Stimulation of atrial receptors by atrial distension is said to inhibit ADH-release while the effect on renal circulation is rather antagonistic in nature i.e. a reflex vasoconstriction. Ventricular receptors sensing the extent of ventricular diastolic filling may also inhibit ADH-release and are capable of inducing particularly marked inhibitions of renal vasoconstrictor fibre discharge (Öberg and Thorén 1975) implying that the hormonal and circulatory responses complement each other in a synergistic way. Reflexes from ventricular receptors may therefore constitute an important volume regulating mechanism.

Gauer J H J Henry and C Behn, *Annual Review of Physiology* 1970 32, 547-595

Öberg B *Acta physiol scand* 1964 62 Suppl 229

Öberg B and P Thorén *Acta physiol scand* 1973 In press

Blarklund, A., Moore, R. Y. and Stenevi, U. (Department of Histology, University of Lund, Sweden, and Departments of Neurology and Anatomy, University of Chicago, U.S.A.): STUDIES ON MORPHOLOGICAL PLASTICITY OF CENTRAL ADRENERGIC NEURONS

The functional plasticity of the central nervous system is well known. A morphological correlate of such plasticity would be a modifiability of axonal connections. and in a series of studies we have explored whether intact catecholamine axons will show alterations in response to a removal of a non-adrenergic input to a terminal region.

Two experimental situations were studied: the adrenergic innervation of the medial and lateral septal nuclei after removal of the non-adrenergic hippocampal afferents to these nuclei, and the adrenergic innervation of the lateral geniculate body after removal of either the non-adrenergic retinal afferents or the non-adrenergic cortical afferents to this region. After removal of the hippocampal afferents to the septum there was a gradual, substantial increase in the adrenergic innervation within 30 days after operation, and this effect persisted up to at least 100 days. The increase in the number of adrenergic fibres was accompanied by a parallel increase in noradrenaline, reaching values of about 200 % of control at 30-100 days. In the lateral geniculate body there was a strong increase in the adrenergic innervation at 13 days after visual cortex ablation (i.e. at a time before the reactive glial changes in the nucleus became prominent). No effect was seen after enucleation, however.

It is suggested that the observed effects are the result of a collateral sprouting of the intact adrenergic axons in response to the denervation, and that the sprouting fibres might reinnervate synaptic sites vacated by the axonal degeneration produced by lesion. If this interpretation is correct, it indicates that intact central adrenergic axons are capable of significant morphological plasticity.

The afferent systems to the hippocampus and fascia dentata are distributed in characteristic and often exclusive laminae. Changes in this laminar pattern are observed following primary unilateral removal of the perforant path fiber input (deentorhination). Rats from 5 to 30 days old had this operation performed. The terminal fields of the remaining systems were studied after various survival times by the Timm method or following secondary lesions and silver-impregnation.

In general the deentorhinated zones of hippocampus and fascia dentata were somewhat shrunken but occupied by abnormal projections of the intact systems. A decrease in the capability of extension of these systems was observed with increasing age at the deentorhination.

With the silver-impregnation method extension of the terminal fields of both the ipsilateral association systems and the commissural systems was found.

The mutual medio-lateral distribution of the extended fibers followed the normal pattern in fascia dentata. The commissural fibers spared the most superficial parts of the deentorhinated zones here.

The Timm method gave corresponding results but revealed furthermore an extension into the outer parts of fascia dentata of a system normally terminating at CA1/subicular transition. This observation is of special interest as this particular part was devoid of commissural fibers. Evidence of a supragranular location of mossy fibers was also found. By the two methods no evidence was found of extension or intensification of the terminal field of septal fibers. An induced acetylcholinesterase rich layer in fascia dentata did not completely correspond with any Timm's staining.

The replacement of the perforant path by other systems is discussed in relation to earlier observations on related phenomena. The possible underlying mechanisms are considered and a definition of the terms aberrant axonal growth, rerouting and sprouting and their characteristics is proposed.

Storm Mathisen J (Norwegian Defence Research Establishment 37
2007, Kjeller, Norway) INCREASE OF CHOLINE ACETYLASE (ChAc)
AND ACETYLCHOLINESTERASE (AChE) IN STRATUM MOLECULARE FASCIAE
DENTATAE FOLLOWING DEGENERATION OF THE PERPHORANT PATH

Previous observations suggested that ChAc activity in the molecular layer of fascia dentata may increase after lesions of the entorhino dentate fibres (Storm Mathisen Brain Res 1972 40 215). To study this the entorhinal area was severed from the hippocampal formation in rats (200 g). Serial horizontal cryostat sections were cut and of each 10-8 were freeze dried, stained for AChE and silver impregnated (Fink Heimer). Degenerating terminals were distributed in the outer and middle zones of the molecular layer of area dentata corresponding to the extent of the lesion (Hjort Simonsen J comp Neurol 1972 146 219). There was an increased AChE staining according to the distribution of degenerating nerve terminals. Samples were dissected from the various zones and assayed for ChAc (Storm Mathisen op cit). ChAc was increased by about 50% in the sites with maximum density of degenerating perphorant path terminals. There was no increase in the zones without such terminals. The increased activities were present at 8 days survival and lasted at least 3 months. They were lost on transection of the septal afferents. It is suggested that the changes may represent a "compensatory" invasion of the vacant synaptic sites by cholinergic nerve terminals. Similar results have been reported for AChE by Lynch et al (Brain Res 1972 42 311).

The large pyramidal cells of the hippocampus (CA3) have axons with two large branches. One branch runs in the fimbria and contacts septal and hypothalamic cells. The other branch curves back into the hippocampal formation, proceeds through the CA3 region and forms a thin layer in the superficial part of stratum radiatum of CA1. In serial electron micrographs the latter fibres, the Schaffer collaterals, have Ranvier nodes with an average internodal length of 75 micron. Most of the Ranvier nodes in CA3 and the adjoining part of CA1 issue 1-3 unmyelinated branches. With intervals of 3-5 micron the unmyelinated fibres show boutons en passage most often contacting dendritic spines, but also shafts of small dendrites. With increasing distance from the CA3 the Schaffer collaterals lose their myelin and proceed as unmyelinated fibres with frequent synaptic swellings.

All 165 CA3 cells tested were antidromically invaded from the fimbria as well as from the Schaffer collaterals in CA1. Both orthodromic and antidromic activation of the Schaffer collaterals gave synaptic waves at depths appropriate with the level of these fibres. The size of the synaptic waves remained fairly constant throughout the distribution of this fibre system, indicating that the number of CA1 cells per area which were synaptically activated remained relatively constant.

In contrast to the behaviour of the fimbrio-septal pathway, the Schaffer collateral-CA1 pathway showed marked frequency potentiation with an optimal frequency around 10-15/second. Responses to single orthodromic volleys along the Schaffer collaterals showed long-lasting (several hours) increase following repeated short tetani (10/sec for 15 sec) to the same pathway. The threshold of the input was not changed but its steepness was increased, indicating that a given volley was able to evoke a larger synaptic potential than before the tetanic priming. These plastic changes may be related to the peculiar synaptic arrangement allowing amplification of the effect of impulses along the Schaffer collaterals. By varying the firing frequency, the CA3 cells may thus bring large or smaller amount of CA1 neurones to discharge, but retaining a relatively constant influence over the fimbrio-septal pathway.

Blood flow to tissues interfacing the vertebrate animal from its environment i gills lungs and skin will be reviewed in a comparative phylogenetical context emphasizing control of ventilation-perfusion matching and skin circulation in the transition from water breathing to air breathing Fish gills receive a cardiac output (CO) ranging from 5 to 40 ml/kg min depending on species temperature and activity At normoxia O_2 uptake varies directly with (CO) Gills possess non-respiratory shunts influencing the effectiveness in O_2 uptake Shunt circulation seems to be under adrenergic control while gas exchange circulation is cholinergic Hypoxia increases gill vascular resistance yet the PO_2 gradient from water to blood decreases showing improved convection of blood and water Heart rate is coupled to ventilatory rate by a vagal reflex mechanism Ventilation-perfusion ratios are high (10-50) due to high ventilation necessitated by the low O_2 content of water Swimming altering distribution of water across gill causes increased blood flow to well ventilated gills and reduced flow to gill with low ventilation Fish myocardium shows higher tolerance to anoxic stress in bottom dwelling species than in species from better aerated water (Poupa & Gesser (pers comm)) Fish using bimodal breathing aquatic and aerial show reflex coupling of ventilation and perfusion of respiratory organs Air breathing increases CO and shifts blood flow from gills to air breathing organ The response is elicited by vagal stretch sensitive receptors in the air breathing organ In facultative air breathing fish (Amia) temperature increase will cause switching from water breathing to air breathing While CO increases linearly with temperature gill vascular resistance drops precipitously when air breathing starts and low resistance bypass of the gills opens up The change is dependent upon a vagal sensory link Inflation dependent perfusion increase to the lung is present in anuran amphibians and reptiles Stimulation of pulmonary vagal stretch receptors greatly lowers pulmonary vascular resistance increases CO and causes a left right shunt in snakes During long respiratory pauses in aquatic reptiles an increasing right to left shunting occurs Such shunting causes a matching of perfusion to O_2 availability in the lung

In aquatic and amphibious species skin circulation is important in gas exchange Both fish living in O_2 deficient waters and amphibians show high density of cutaneous capillaries Vasoconstrictor mechanisms (neural and direct) cause skin vasodilatation under various conditions e.g. hypoxic water (fish) prevention of air breathing (lungfish) CO_2 breathing pulmonary obstruction and diving (amphibians) In reptiles skin circulation is less important in gas exchange but has become important in temperature regulation It has been shown that skin blood flow (lizards turtles) increase during heating and decrease during cooling independent of body temp (BT) if the latter is below preferred BT A right left shift and increased heart rate and sympathetic renal vasoconstriction are factors causing increased skin flow during heating The importance of skin circulation in thermal homeostasis is crucial in mammals and birds possibly maximal in birds from polar regions Skin of birds feet (*Macropodops giganteus*) shows peak blood flows >200 ml/kg min promptly onset on cold immersion Skin vasoconstriction and dilatation can be mimicked by stimulation of cut peripheral nerves suggesting presence of neurogenic vasodilator mechanisms Skin vascular smooth muscle shows adaptive change in sensitivity to vasoactive agents at lowered tissue temperature

Previous studies of the primary somesthetic cortex (S I) in anesthetized monkeys have indicated a mediolateral somatotopic progression of receptive fields and a segregation of modalities such that neurons with cutaneous receptive fields are more common in the anterior part of the postcentral gyrus, and neurons of deep modality are more common posteriorly. In awake behaving monkeys we have confirmed these findings, but we have also seen evidence for convergence of different modalities to cells that form cortical columns in the posterior part of S I and the parietal associative area (Brodmann's area 7).

Awake, sitting stump-tail monkeys were studied with the transdural microelectrode recording technique developed by Evarts. Peripheral receptive fields in the hand area of S I were mapped using tactile stimuli and manipulation of joints and muscles.

Cells with simple skin fields were the majority of those studied in all the three cytoarchitectural areas of S I (Brodmann's areas 3, 1 and 2 from anterior to posterior). However, in a sample of 581 histologically localized cells studied in S I at the time of writing this abstract, the percentage of simple skin field neurons decreased from 70 % in area 3b to 63 % in area 1 and 33 % in area 2. Further posteriorly in the parietal area 7 this percentage was only 11. Whereas the number of simple skin fields decreased posteriorly, the number of more complex receptive fields increased in the same direction. Cells that required a particular direction of movement along the skin, a particular orientation of a tactile stimulus edge or that were activated both from skin receptors and deep (joint or muscle) receptors were only 10 % of the total in area 3b. In area 1 their number was 18 %, in area 2 it was 35 % and in area 7 they appeared to constitute well over half of the cells, but in that region the cells receive visual input, too. These proportions must be considered tentative until more precise methods for identification of the input receptors are used, but these percentages show a gradual change from simple receptive fields anteriorly in S I to more complex activating connections in posterior S I and adjacent parietal associative area.

The most prominent difference between S I and the adjacent parietal associative area is the visual activation in area 7 which we have not seen in S I. The visual activation in area 7 is related to eye movements in a particular direction or to a particular direction of gaze. Moreover, manipulative movements of the hand in the same direction often activate cells in area 7 also when vision is blocked. Thus cells in this region are truly "associative" between two different modalities, being activated by both somesthetic and visual mechanisms related to a particular direction in the space around the animal.

This work has been supported by the Foundations' Fund for Research in Psychiatry.

Although primary (essential) hypertension has initially a circulatory pattern similar to a mild defence reaction its established phase is characterized by a raised systemic flow resistance. It has been widely assumed that this increased resistance is due to a raised vascular smooth muscle activity. Consequently the intense study of the pathogenesis of this common disorder of regulation has concentrated on the origin of this increased smooth muscle tone. The debate has centred inconclusively on neurogenic, hormonal and ionic factors and/or on an increased effector sensitivity to such influences.

The problem has been approached from an entirely different angle in studies based on haemodynamic analyses in man and spontaneously hypertensive rats (SHR). These results (see Folkow 1971) strongly indicate that in both types of primary hypertension the raised resting resistance is essentially the result of an increased arterial media thickness, partly encroaching upon the lumen even at maximal dilatation. This implies a normal structural adjustment to increased functional load similar to hypertrophy of the left ventricle and of arterial walls in general (cf Suwa and Takahashi 1971). Thus even at maximal dilatation precapillary resistance is raised above control values. Moreover, for given smooth muscle activations exaggerated luminal reductions ensue, resulting in a raised resistance and vascular hyperactivity even at normal levels of smooth muscle activity. Also the important baroreceptor re-setting (cf Aars 1969) appears to be mainly a consequence of such a vascular wall adaptation.

Studies on rats show that this structural adaptation is such a rapid process that it becomes closely interwoven in time with functional triggering factors which in SHR are at least partly constituted by the neurohormonal excitatory pattern commonly induced by increased alertness or alarm (the defence reaction). When often repeated such reactions lead to a gradual rise in resting pressure even in rats that are not genetically predisposed to hypertension. The greater the average pressure load, the stronger is the tendency for structural adaptation of the heart and precapillary vessels. Comparisons between SHR and normotensive and renal hypertensive rats suggest that SHR display exaggerated defence reactions to standardized stress situations and further that their heart and vessels are somewhat more prone to adapt structurally to a given pressure, presumably forming part of the polygenetic background of their hypertension. Parallels to man suggest that similar principles might contribute to the hereditary background of essential hypertension, as reinforced by the extrinsic drive constituted by stressful environmental influences. Hypotensive treatment by drugs or other interferences can cause rapid regression of the structural changes and may, when used e.g. for prevention in prehypertensive SHR, largely hinder the development of both hypertension and structural vascular changes.

Ref Folkow B Clin Sci 1971 41: 1-12

Suwa N and Takahashi T Morphological and Morphometrical Analysis of Circulation in Hypertension and Ischemic Kidney Urban & Schwarzenberg München-Berlin-Wien 1971

Aars H Acta physiol scand 1969 73: 406-434

The discharge from muscle spindles in the antibrachial finger flexor muscle of conscious human subjects was analysed when the muscles were relaxed during isometric voluntary contractions and during active movements. The spindle discharge increased with muscle length in relaxed muscles and was largely constant with time after an adaptation period of 5-15 sec. At a comfortable resting position of the hand only five percent of the spindle primaries were continuously discharging their impulse frequency did not exceed 20 ips (impulses per sec) and their sensitivity to muscle length was below 1.0 ips/mm. The low discharge rate and the low sensitivity in comparison with other preparations indicate that the fusimotor outflow is negligible to relaxed muscles and any functional role of the poor afferent activity under these conditions may be questioned altogether. No dramatic changes in spindle afferent discharge could be provoked by noxious or non-noxious skin stimuli by varying the input through the neck reflex afferents or to the vestibular organs as long as the muscles were relaxed. Thus there were no functionally significant modulations of the fusimotor activity in this preparation by a number of procedures which have been shown in animal experiments to modify the fusimotor outflow.

During voluntary contractions on the other hand there was a powerful fusimotor outflow which largely ran parallel with the skeletomotor outflow in spatial temporal and intensive respects. This implies somewhat unexpectedly that the basic pattern of intrafusal activation is very similar in the human hand and in the much more primitive system of amphibians the design of which entails an obligate and rigid co-activation and parallelism of the extrafusal and intrafusal muscle activity. This suggests that the significant role of the separate fusimotor system in higher animals is to refine the performance rather than to alter the basic function of the muscle spindle system.

A closer analysis of the temporal relations at the onset of voluntary contractions revealed that the contractions were all initiated by the skeletomotor neurones whereas the rest of the contractions involved the joint activation of the two motor systems in fast as well as in slow contractions. This shows that the command signal to the muscle is not delivered to the gamma motor neurones alone and therefore the follow up length servo hypothesis must be rejected.

During active movements many spindle primaries signalled the speed of movement quite accurately. The reflex effects of this afferent activity on the spinal level would be to eliminate variations in the speed of movement and thus to make the active movement progress more smoothly and fluently. Although this function may represent but a limited facet of the role of the muscle spindles the findings suggest that the main significance of this complicated system is during contraction and in regulating the characteristics of active movements.

Recent results demonstrate that the pH of bulk cerebrospinal fluid (CSF) equals that of the cerebral extracellular fluid (ECF). Since the ECF pH serves as an important regulator of pulmonary ventilation and a major determinant of cerebral blood flow many attempts have been made to unravel the physiological mechanisms which are responsible for the control of the CSF pH. These mechanisms still remain largely unknown and several possible regulatory factors will therefore be described and examined.

The CSF (and ECF) is a bicarbonate-containing fluid which contains very little protein or other non-bicarbonate buffers. Its buffer capacity to CO_2 is therefore low and the HCO_3^- concentration does not vary significantly when the CO_2 tension is altered in vitro. It follows from this that variations of CSF HCO_3^- concentration in vivo are due to ion exchanges with either blood or tissue cells.

At steady state and under normal acid-base conditions the CSF HCO_3^- concentration and the CSF pH are lower than in plasma water. This relative acidosis in CSF is maintained in spite of the fact that the d.c. potential between CSF and plasma is positive (about 4 mV at a plasma pH of 7.40). The net electrochemical potential differences for H^+ and HCO_3^- ($\Delta\mu$) cannot be explained by the fact that the CSF is secreted as an acid fluid. In fact the results obtained by Maren suggest the opposite, i.e. that the newly formed CSF contains a high bicarbonate concentration. There are two possible mechanisms which could maintain the net $\Delta\mu$ for H^+ : (1) active transport of H^+ from plasma to CSF or (2) efflux of acids from the tissue cells.

In respiratory acidosis there are relatively rapid increases in the CSF HCO_3^- concentration which limit the fall in pH. The mechanisms involved cannot achieve complete normalization of the CSF pH but operate even if the plasma HCO_3^- concentration is kept constant. In non-respiratory acidosis and alkalosis the CSF HCO_3^- concentration varies much less than the plasma HCO_3^- concentration even in chronic cases and the CSF pH shows a remarkable constancy. Calculations of electrochemical potential differences show that the $\Delta\mu$ values are essentially unchanged and thus give little support to the hypothesis of an active transport regulation of CSF pH. The CSF/plasma d.c. potential varies inversely with the plasma pH even in chronic acid-base changes and it remains a possibility that the regulatory changes in the HCO_3^- ratio between CSF and plasma are at least partly caused by the changes in d.c. potential.

Hyperventilation elicited by hypoxia is accompanied by an efficient regulation of the CSF pH. However if one assumes that the hypoxia leads to increased acid production in the brain the pH homeostasis may reflect respiratory regulation of a primary acidosis of nonrespiratory nature. In nonrespiratory acidosis and alkalosis an efficient regulation of the CSF pH occurs only when a compensatory change in pulmonary ventilation can occur. Since neither respiratory acidosis nor passive hyperventilation is accompanied by complete or near-complete restitution of the CSF pH, it appears that the pronounced regulation of the pH only occurs in nonrespiratory conditions that allow ventilatory adjustments.

44 Thealeff S. (Department of Pharmacology University of Lund Sweden):
CHOLINERGIC RECEPTORS IN MAMMALIAN SKELETAL MUSCLE

In innervated mammalian skeletal muscle acetylcholine (ACh) receptors are present in great density about $10^5/\mu m^2$ at the end plate region while their density at extrajunctional sites is less than $5/\mu m^2$. Following denervation the receptor density at the end plate is maintained but that of the extrajunctional membrane is increased to about $10^2/\mu m^2$.

When extrajunctional cholinergic receptors appear the rate of rise of the action potential is markedly reduced and the spike is followed by a long-lasting positive afterpotential. At the same time the action potential becomes resistant even to high concentrations (10^{-6} - 10^{-7}) of tetrodotoxin (TTX). The appearance of TTX-resistant action potentials coincides spatially and temporally with the increase in the number of extrajunctional cholinergic receptors.

The induction of extrajunctional cholinergic receptors and of TTX resistant action potentials in denervated muscles is prevented by the administration of actinomycin D to the animal. However, once the denervation changes are established actinomycin has no effect on these membrane properties indicating that their induction depends on genetically induced protein synthesis.

In view of the temporal and spatial correlation between the appearance of extrajunctional cholinergic receptors and of TTX resistant action potentials it has been speculated that structural proximity or identity between the two types of ionophores may exist in the denervated muscle membrane.

The application of colchicine or vinblastine, drugs known to block axoplasmic flow to the sciatic nerve of the rat induce the appearance of extrajunctional cholinergic receptors and of TTX resistant action potentials in leg muscles. This occurs without affecting nerve impulse transmission, no paralysis of the hind leg is observed and the twitch as well as the tetanic tension of the muscle is intact. Neither is spontaneous transmitter release affected by the drugs. It is concluded that lack of substances normally carried by axoplasmic transport induce the appearance of extrajunctional cholinergic receptors and of TTX resistant action potentials in denervated muscle. This conclusion is supported by the observation that the time of onset of the denervation changes depends upon the length of the nerve stump distal to the site of neurotomy.

When studying the transport of solutes and water across epithelia one can use the black box approach and describe the processes in terms of active transport of certain substances which in turn by electric or hydraulic coupling may contribute to the transport of passively transported substances

In recent years however the interest has been switched towards a resolution of the generalized fluxes across the epithelia into flows through specific pathways

It has become apparent that epithelia can be of a high resistance and a low resistance type. Formally the epithelia of the latter type (e.g. gall bladder, small intestine, proximal kidney tubule) differs from those of the former type (frog skin, urinary bladder, large intestine etc.) in that they possess a pronounced shunt pathway which is rather indiscriminately permeable to small ions and small hydrophilic molecules. Hyperosmolarity on the mucosal side of several tight epithelia transforms them reversibly into the leaky type. Evidence coming from several laboratories including our own indicate that the main shunt pathway in the leaky or low resistance epithelia is intercellular through "leaky seals". The leaky seals have been visualized for instance by precipitation of BaSO_4 (Ussing H Phil Trans Roy Soc Lond B 1971 262 85) or lanthanum (Martinez-Palomo A, Eriij D and Bracho H J Cell Biol 1971 50 277) or they have been localized electrically for instance by probing the surface of the epithelium with a micro electrode (Frömter E and Diamond J Nature 1972 235 9).

Another type of pathway is provided by the coupling between neighbouring cells through low resistance cell contacts. Present data indicate, however, that in the case of say frog skin the coupling between cells in different layers of the epithelium if existent must be through rather high electric resistance paths. This conclusion is based on the volume changes in different layers in response to electric current flow in the inward and outward directions (Voite M and Ussing H Exp Cell Res 1970 61 133).

The polar behaviour of epithelia stems from two independent sources: 1) The fact that the tight or leaky seals are placed asymmetrically, mostly near apical end of the mucosal side of the epithelia and 2) the fact that the inward and outward facing cell membranes of the epithelium differ with respect to passive and active transport pathways

The major difficulty in using the sliding filament model to explain contraction in vertebrate smooth muscles has been that here the presence of myosin in a filamentous form could not be established unambiguously. This was achieved only recently by X-ray experiments with living muscles (for references see Lowy et al Phil Trans Roy Soc 1973 265 191). Such experiments also provided further evidence for the operation of a sliding filament mechanism by showing that during contraction the axial periodicities due to the arrangement of the actin and myosin molecules remain practically constant. A number of important problems remain unsolved. One of them is whether the filamentous myosin elements occur in the form of ribbons or rods. Another concerns the arrangement of the actin and myosin filaments in the contractile apparatus. Both problems are at present the subject of a lively controversy. From their X-ray and electron microscope work Lowy et al believe that myosin is present in unique ribbons which are randomly disposed in the cell. On evidence from electron microscopy Somlyo et al (for references see Phil Trans Roy Soc 1973 265 233) propose a structural picture which resembles more closely that seen in striated muscles namely rod-like myosin filaments arranged in a regular lattice.

At present there are great difficulties in the way of solving the ribbon-rod problem. We have concentrated on an X-ray technique that uses a high-resolution double-monochromator camera of the type described by Huxley & Brown (J Mol Biol 1967 9 383). This enables us to determine some of the dimensions

the myosin elements in living muscles. Results obtained by Lowy, Poulsen & Vibert show that in the guinea pig's taenia coli muscle maintained in a cold hypertonic Ringer solution the myosin cross bridges are in register over a distance of about 1300 Å laterally (lateral coherent unit = LCU). In muscles kept in cold isotonic Ringer the LCU is less than 1300 Å but at least 800 Å. These figures exclude the possibility of diffraction from individual rods which (according to Somlyo et al) have a diameter of at most 200 Å plus a halo region that surrounds each rod and extends to no more than another 200 Å. But these rods were visualised by fixation of muscles in warm isotonic Ringer and from our X-ray results to date we cannot give a figure for the LCU of muscles kept under such conditions. What we have established is that in cold hypertonic Ringer, myosin elements aggregate to form structures which are larger than those present in cold isotonic Ringer.

We have as yet no evidence from studies of the equatorial diffraction pattern that the myosin elements (under any conditions) are organised in a regular sideways arrangement like the one seen in the electron micrographs of Somlyo et al.

Monoaminergic neurons maintain their transmitter stores relatively constant in spite of considerable variations in physiological activity suggesting that transmitter synthesis is promptly adjusted according to the needs. Induction of the rate-limiting synthetic enzyme tyrosine hydroxylase occurs only after a latency of several hours. The increased transmitter synthesis demonstrated after short-term stimulation is therefore presumably due to an activation of existing enzyme molecules. It has been proposed that this short-term adjustment is mediated via changes in end-product inhibition, but such inhibition requires relatively high catecholamine concentrations (about 10^{-4} M) and is thus of doubtful physiological significance.

Two examples will be given where short-term regulation of tyrosine hydroxylase activity *in vivo* occurs independently of release by nerve impulse and where end-product inhibition does not seem to be involved.

1) In the adrenal medulla of the rat dopamine (DA) mainly if not entirely serves a precursor role constituting about one per cent of the total catecholamines. Neurogenic stimulation of the gland by means of drugs acting via supraspinal centres causes a considerable increase in DA occurring in spite of a marked acceleration of DA turnover and thus presumably due to an increased synthesis caused by an activation of tyrosine hydroxylase. Chlorisondamine, a ganglionic blocking drug inhibits the release of adrenomedullary hormones efficiently but leaves the elevation of DA unaffected. As indicated by turnover data this elevation is due to an increased synthesis. The effect is abolished by transection of the spinal cord. — Thus the tyrosine hydroxylase activity of the adrenal medulla can be promptly adjusted via a neurogenic mechanism operating at least partly independently of the physiological release mediated via nicotinic receptors.

2) If the nigrostriatal DA axons of the rat are cut the striatal tyrosine hydroxylase activity *in vivo* is markedly elevated during the first 30–60 min. This paradoxical response is presumably due to a feedback mechanism mediated via postsynaptic (or possibly hypothetical presynaptic) DA receptors. Evidence for this hypothesis has been obtained by demonstrating changes in the tyrosine hydroxylase activity *in vivo* after the administration of DA receptor agonist (apomorphine) and an antagonist (haloperidol) either separately or combined. — It should be noted that this control mechanism is capable of operating in the absence of an impulse flow.

These prompt and marked adjustments of tyrosine hydroxylase activity probably occur without any concomitant changes in the synthesis of macromolecular cell constituents and are thus difficult to reconcile with the concept that the release by nerve impulses occurs exclusively through exocytosis.

48 Rosell, S. (Department of Pharmacology Karolinska Institutet, Stockholm Sweden): VASCULAR REACTIONS AND MOBILIZATION OF FAT IN ADIPOSE TISSUE

It is reasonable to assume that the blood circulation in adipose tissue is well adapted for the specific function of providing an efficient transport of fat. The adrenergic neuro humoral system seems to be of prime importance in the control of circulation and lipolysis in adipose tissue. The lipolysis leads to an outflow of free fatty acids (FFA) and glycerol.

From available data in dogs some characteristics can be established and compared with other vascular circuits as e.g. skeletal muscle. Resting blood flow in adipose tissue is 6-9 ml/min/100g. The maximal blood flow produced by vasodilating agents (about 40 ml/min/100g) is less than the corresponding value for skeletal muscle. This seems to imply smaller dimensions of the arterial system in adipose tissue. On the other hand resting and maximal values for the capillary filtration coefficient (CFC) are larger than in skeletal muscle. The density of the capillary network surrounding every adipocyte is extensive. In addition morphological studies indicate that the adipocytes at least in the mesentery are located toward the venous section of the capillary bed rather than to the arterial side. This indicates that the adipocytes are localized near vessels with the highest permeability. These data indicate that favourable conditions for capillary exchange exist in subcutaneous adipose tissue.

Stimulation of the appropriate sympathetic nerve to canine subcutaneous adipose tissue produces adjustments in all the series coupled sections of the vascular bed i.e. constriction of resistance and capacitance vessels and a net transcapillary fluid absorption. A conspicuous difference from such adjustments in other tissues is a marked increase in CFC during stimulation sometimes to three times the basal value. In addition the isovolumetric capillary pressure (P_{ci}) increases.

These data indicate that increased neuro humoral activity may increase the capillary permeability. This increase is concomitant with a decreased capillary surface area as indicated by a diminished disappearance of xenon from a local tissue depot despite a constant total blood flow. The described adjustments of the capillary bed may promote the outflow of the product of lipolysis without a simultaneous outward fluid filtration.

The lipolytic rate is enhanced by increased neuro humoral activity. Quantitative comparisons indicate that the innervation of adipose tissue is more important for the control of the lipolysis than the circulating catecholamines. However there are regional differences in the importance of the sympathetic neuro humoral control of lipolysis. Perhaps hormones rather than the sympathetic system play a primary role in some regions. This possibility has not yet been evaluated experimentally.

Intestinal blood flow represents one factor of importance for intestinal absorption in vivo, although blood flow in the mucosal layers has been considered to be normally so large as to never limit the rate of intestinal absorption. However the demonstration of a countercurrent exchanger in the intestinal mucosa has greatly complicated the seemingly simple relationship between blood flow and absorption. Anatomically this countercurrent exchanger is constituted by the arterial supply to each villus as surrounded by the dense subepithelial capillary network in which flow is countercurrent to arterial inflow. The distance between the two limbs of these hairpin vascular loops of the villi is only 10-20 μ m. This vascular arrangement has functional implications that will now be briefly summarized (cf Lundgren Acta physiol scand, Suppl 303).

Absorption of easily diffusible lipophilic solutes. Mean transit time of plasma particles in the villous vascular loops amounts to 3-5 sec at resting blood flow in cat small intestine. 9/10 of this time being spent in the subepithelial capillary network. Since most lipid soluble substances equilibrate almost completely in a fraction of a second between the two vascular limbs of the countercurrent exchanger absorbed lipophilic solutes may easily diffuse from capillary to arterial vessel along the concentration gradient established upon absorption. The solute is then brought back towards the tip of the villus and hence "hindered" from being absorbed. Such a delaying effect on intestinal absorption has been demonstrated in model experiments studying ^{85}Kr -absorption (Svanvik Acta physiol scand Suppl 385).

Absorption of fatty acids. It is well known that long chain fatty acids are absorbed mainly via the lymph while short chain ones are transported mainly in the portal blood. These different absorption routes have been explained in terms of a rapid esterification in the epithelial cells of long chain but not of short chain fatty acids. The partition between lymph and blood for different fatty acids may however also be explained by the countercurrent exchanger acting as an efficient hindrance to net blood absorption of mainly the lipophilic long chain fatty acids in a way similar to that described above for other lipophilic solutes. These

"trapped" fatty acids are then eventually built into triglycerides in the epithelial cells. The water soluble short chain fatty acids on the other hand are far less easily affected by the exchanger since they are pore-bound when passing the vascular endothelium. Recent experimental observations strongly support this hypothesis (Jodal. The significance of the intestinal countercurrent exchanger for the absorption of sodium and fatty acids. Göteborg 1973).

Absorption of sodium and water. The intestinal absorption of solutes and water is closely linked and it is assumed that a difference in osmolarity across the intestinal epithelial barrier is a prerequisite for the transepithelial transport of water. This hyperosmolar region is according to current theories localized to the epithelial intercellular spaces. However recent findings favour the view that the villous countercurrent exchanger functions as a countercurrent multiplier during absorption of e.g. sodium chloride creating a interstitial hyperosmolarity which in the villous tips reaches at least 600 mOsm/l. This hypothesis also implies that the initial water absorption to a large extent takes place via the villous lymphatics.

Osmolar changes in the organism regional as well as more generalized ones can affect cardiovascular hemodynamics profoundly via marked influences on vascular tone fluid distribution between compartments and plasma volume. Osmolar control mechanisms seem of special importance for the circulatory adjustments in muscle exercise and in certain pathophysiological states such as hemorrhagic hypotension.

In exercise pronounced tissue hyperosmolality develops in the interstitial space of active muscle due to production and release of osmotically active particles from the contracting striated muscle fibres. The interstitial hypertonicity leads to osmotic shrinkage of the vascular smooth muscle cells and to changes in transmembrane ionic concentration gradients and membrane permeabilities which cause inhibition of vascular tone. Tissue hyperosmolality acts thereby as one of the dominating mediators of functional hyperemia in muscle and it also improves capillary flow distribution and exchange in the tissue (Mellander S et al *Angiologica* 1967 4 310; Lundvall J *Acta physiol scand* 1972 suppl 379). A similar mechanism may contribute to the vasodilation in the myocardium and sweat glands during work. Exercise further leads to a very rapid exudation of plasma fluid into the extravascular space of the active muscles mainly (> 75 %) due to osmosis caused by the work-induced tissue hyperosmolality but also to filtration caused by increased capillary pressure. In heavy short-term whole-body exercise in man more than 1 litre of plasma fluid is lost to the active muscles. Yet the plasma volume reduction in the exercise period is kept within tolerable limits owing to concomitant absorption of some 500 ml of extravascular fluid from inactive tissues. This compensatory fluid gain is partly caused by osmosis due to a pronounced generalized arterial hyperosmolality which results from overflow of osmoles from the active muscle mass and is partly caused by a reflex decrease of capillary

(Lundvall J et al *Acta physiol scand* 1972 85 258). The arterial hyperosmolality in exercise also seems to promote the action of heart via an inotropic effect (Wildenthal K et al *Amer J Physiol* 216 898) and to decrease pulmonary vascular resistance and concomitantly constrict the pulmonary veins so as to mobilize blood for the systemic circulation (Björk G et al *Acta physiol scand* 1971 82 375). The reflex increase of blood pressure and the tachycardia in exercise in part may be attributed to stimulation of local osmoreceptors in active muscle (Lasser R. et al *Circulat Res* 1960 8 913).

In hemorrhage there is also an important compensatory osmolar control of plasma volume via transcapillary fluid absorption caused by pronounced arterial hyperosmolality. In this case however the arterial hyperosmolality is related to hypoglycemia caused by glucose release from the liver (Jörhult J et al *Acta physiol scand* 1972 85 142).

Fluid escape into the extravascular space seems inevitable when tissue hyperosmolality develops. It occurs besides in active muscle in glands during secretion and in certain pathophysiological states such as second degree burns (Arturson G and Mellander S *Acta physiol scand* 1964 62 457). Fluid movement into the interstitial space may help to improve tissue nutrition since such fluxes have been found to augment the capillary transfer of solutes (Lundgren B and Mellander S *Acta physiol scand* 1967 70 26).

Irvine D R F and Wester K G Department of Psychobiology
University of California (Irvine): MIDDLE EAR MUSCLE EFFECTS ON COCHLEAR
RESPONSES TO BONE-CONDUCTED SOUND

Contractions of the stapedius and tensor tympani muscles were elicited by electrical stimulation of their motor nerves or of the muscles themselves in anaesthetized cats. The effects of these contractions on cochlear microphonic responses to air- and bone-conducted sound were examined. Stapedius contractions that produced changes in air conduction similar to those observed under physiological conditions had almost identical effects on bone-conduction. Tensor tympani effects on bone-conduction were of similar magnitude but greater complexity than those on air and varied as a function of the location of the bone-conductor on the skull. Control observations established that the effects were attributable to the middle ear muscles and not to other consequences of the experimental procedures and that they did not reflect modification of an air-conduction component of the bone-conduction stimulus. The functional significance of these effects is discussed in terms of protection against masking of environmental sounds by self-generated bone-conducted sound. It is concluded that the middle ear muscles may subserve such a protective function.

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SoviĖrvi A R A, (Institute of Physiology, University of
Helsinki, Finland): DETECTION OF TEMPORALLY AND SPATIALLY
COMPLEX ACOUSTIC SIGNALS BY CELLS IN THE CAT PRIMARY AUDITORY
CORTEX

The neural mechanisms involved in detection of complex and moving sounds were studied by recording single-neuron responses from 132 cells in the cat primary auditory cortex. The cats were paralysed and under neuroleptanalgesia. The responses evoked by pure tones and by many types of natural complex sounds were compared with each other. 25 cells mainly giving inhibitory and phasic responses to pure tones were selected for tests with moving sounds. Excitatory, inhibitory and phasic components in the pure tone responses were almost equally common. Phasic-response cells generally reacted in a time-locked fashion to transient complex sounds as could be predicted. Non-predictable responses to complex sounds were given by 32 per cent of the cells; some cells responded in quite a different way (e.g. excitatory contra inhibitory) to complex sounds than to pure tones, and some others (17 %) responded only to certain complex sound patterns. From 25 cells tested with moving sounds 8 responded selectively to the direction of the movement of the sound source. The findings indicate that the primary auditory cortex contains cells, which may have a specialised function in detection of complex sound structures and the direction of sound source movement.

- 53 Van Essen D and Kelly J (Department of Neurobiology Harvard Medical School Boston Mass USA) STRUCTURAL AND FUNCTIONAL PROPERTIES OF SINGLE CELLS IN AREA 17 OF THE CAT

Most neurons in area 17 of the cat can be classified as stellate or pyramidal on morphological grounds and as simple complex or hypercomplex on the basis of their responses to visual stimuli. We have used the technique of intracellular dye injection to study the relationship between the anatomy and physiology of these cells. Microelectrodes filled with Procion yellow were used to stain single neurons whose receptive fields were mapped with spots and slits of light. Forty nine cells have been successfully injected and identified. Most of the simple units were stellate (8 of 12) while pyramidal cells constituted the majority of complex cells (19 of 28) and hypercomplex cells (5 of 7). One cell with intermediate functional properties was pyramidal. Several of the injected complex cells were neither stellate nor pyramidal but belonged to more infrequently occurring neuronal types such as multiform and double bouquet cells. Simple cells occurred most frequently in layer IV of the cortex; complex units were aggregated in both superficial and deep laminae and hypercomplex cells were concentrated in layers II and III. These results indicate that there is a close correlation between the structure and function of individual neurons in the visual cortex. (Supported by NIH Fellowships NINDS 5 P02NS51986-01 and NEI 1 P02 EYS1537-01 and NIH Grant Nos 5 R01 EY00605 13 and SRO1 EY00606 08)

- 54 Poranen, A. (Institute of Physiology, University of Helsinki, Finland): MULTIPLE UNIT AND EEG ACTIVITY IN DIFFERENT SOMATOSENSORY AREAS OF BEHAVING MONKEYS IN A VIBRATION DETECTION TASK

The roles of different structures in the somatosensory system in various somatosensory tasks have remained equivocal in spite of many ablation studies. Therefore, multiple unit and EEG recordings were made in different somatosensory areas of behaving monkeys taught to attend to a cutaneously presented vibratory signal or to ignore it.

In the VB complex of the thalamus vibration causes a clear response which is sometimes modified by attention. The cell groups in areas 3, 1 and 2 responded well to vibration but attention had only slight effects. Vibration also activated parts of S II and motor cortex and sometimes attention had an effect, too. In area 7 there were found no responses to vibration. In all areas mentioned there were cell groups which did not change their response with attention.

The results indicate that attention to vibration can modify the activity of some cell groups in different somatosensory areas. Thus these areas are obviously participating in the sensory-motor interaction that takes place during the performance of the task. The numerous recordings in which attention had no effect on the cellular activity indicate that the interaction may occur on lower levels of the central nervous system, too.

Edwall L, G Ha gerstam and L Olqart (Department of Pharmacology Karolinska Institutet Stockholm Sweden): EXCITATION OF INTRADENTAL SENSORY UNITS AND CHOLINERGIC RECEPTORS 53

Physical stimuli such as air blasts drying and reduced pressure applied on exposed dentin in human teeth produce pain. These stimuli also give rise to afferent nerve activity from the teeth of the cat. Morphological studies have shown the presence of cholinesterase in sensory nerves in the dental pulp as well as structure resembling a synaptic cleft between sensory nerve endings and odontoblasts (Arwill & Lilj. Oral Physiol. Pergamon Press 1972 Arwill Odont. Rev 1967 18 191) This led to the question of whether or not acetylcholine is a mediator in dental pain transmission upon physical stimuli.

Nerve impulses from the pulp were recorded by means of low impedance electrodes inserted in dentinal cavities in the tooth of the cat (Edwall & Scott Acta physiol. scand 1971 82 555). An air blast proved to be the most efficient physical stimulus to excite the intradental nerves. Local application of acetylcholine caused a stimulus response. This response to acetylcholine was followed by a transient blockage to repeated application. The response to acetylcholine could be blocked by di-tubocurarine, atropine and hexamethonium administered locally. In contrast, the response upon physical stimuli could not be blocked by the anticholinergic drug. Moreover, after application of acetylcholine and during the period of depression to acetylcholine the preparation responded to physical stimuli. These findings suggest that acetylcholine is not a mediator in the intradental pain transmission upon physical stimuli.

Knibestål, M. (Department of Physiology Biological Institute University 36 of Umeå, Sweden): QUANTITATIVE STUDIES ON SLOWLY ADAPTING MECHANORECEPTORS IN THE HUMAN GLABROUS SKIN AREA.

It has recently been shown that two types of rapidly adapting mechanoreceptors in the human glabrous skin area (RA-receptors and PC-receptors) have a stimulus response relationship that can be best described by a log hyperbolic tangent function and not by a simple power function as has generally been reported for cutaneous mechanoreceptors in many animal species (Knibestål M. J Physiol 1973 to be published).

Stimulus-response functions have now been analysed for a number of slowly adapting cutaneous mechanoreceptors in the same area (type SA-I and type SA-II). The results seem to indicate that for these receptors it may be of importance what measure is used for the nervous discharge. If the nervous discharge is expressed as the total number of nerve impulses evoked by a stimulus, the stimulus response curves have a sigmoid shape with a lower purely dynamic part and an upper static part. In this case the log hyperbolic tangent function is the best mathematical description of the curves over the total range of stimulus intensities whereas a power function is a reasonable description only for the static part of the curves. If as a measure of the nervous discharge is used the mean impulse frequency of the sustained discharge, the log hyperbolic tangent function is still the best description for the majority of units whereas the power function is the best description for a few units.

It is concluded that stimulus response relations for human cutaneous mechanoreceptors in general are best described by non-power functions.

57 Møntanarta, H (Max-Planck-Institut für Verhaltensphysiologie Seewiesen, V-Tyrol): THE OLFACTORY SENSE OF THE PINE SAWFLY (HYLOBIUS BIEY)

The possibility of using olfactory attractants in controlling pest insects has increased the importance of investigating their olfactory sense. The pine sawfly causes great damage to European forests and the question is which olfactory attractants are involved in its orientation.

Electron microscopical studies have revealed two main types of olfactory sensilla localized to different fields of the antennal club. Each armillum is innervated by one or two sense cells. The function of these cells is studied electrophysiologically. By recording extracellular spikes from single cells while stimulating with different odours up to 200 a response spectrum for each cell was obtained. The most potent stimulating agents were found within naturally occurring mono- and bicyclic monoterpenes.

The response spectra indicate that these two terpene groups interact mainly with their respective acceptor types. Since these acceptor types are situated in different cells discrimination between these terpenes is assumed to take place on receptor level. However, overlap of response spectra for other cells within these substances suggest an additional central nervous evaluation in the discrimination between them. This seems to be the case for the bicyclic terpene pinene and the bark beetle pheromone frontalin which are two of the most important of the tested compounds with respect to response degree and specialization of the cells. Preliminary behavioural observations indicate that these two substances are also important for the orientation of the animal.

58 Døving, F. B. (Institute of Zoophysiology University of Oslo)
Nordeng, H. (Zoological Laboratory University of Oslo)

Oakley, B. (Department of Zoology Michigan University Ann Arbor, USA) Russwurm, H. (present address Norwegian Food Research Institute As) SKIN MUCUS BASIS FOR ODOUR DISCRIMINATION IN MIGRATING CHAR (*Salmo alpinus* L.)

Nordeng (1971) has provided evidence that migrating char locate their spawning ground by pheromone trails produced by the young river population. Extracellular recording of single unit activity in the olfactory bulb of these fish demonstrates that different char populations evoke different reaction patterns. Thus a physiological basis for the pheromone hypotheses is given. Skin mucus of one fish most frequently evoked the same kind of responses as did water in which this fish had been swimming. It therefore seems probable that the skin mucus contains the substances responsible for the physiological responses in the bulbar neurones. In attempts to identify the active substances samples of skin mucus were fractionated in a Sephadex G 25 column with distilled water as effluent. The fractions were tested on the fish while recording the gross bulbar activity. Peak activity was obtained for one fraction only. Different mucus samples gave peak activity with different fraction numbers.

Nordeng, H. (1971) Nature 233 411-413

Lindvall O and A Björklund (Department of Histology University of Lund Lund 59 Sweden); GLYOXYLIC ACID CONDENSATION A NEW FLUORESCENCE HISTOCHEMICAL METHOD FOR BIOGENIC MONOAMINES AND RELATED COMPOUNDS

Glyoxylic acid (GA) has a considerably higher capacity than formaldehyde (the reagent in the Falck-Hillarp technique) to form fluorophores with biogenic indolamines and catecholamines. Strong fluorescence is induced also from N-acetylated indolamines (e.g. melatonin), methoxylated catecholamines (e.g. 3-methoxytyramine) and peptides with tryptophan in NH₂- or COOH-terminal position; these compounds cannot be visualized with the standard Falck-Hillarp technique. The fluorophores formed from primary and secondary phenylethylamines and indolamines in the GA reaction are strongly fluorescent isoquinoline and β -carboline derivatives.

The GA method has been successfully applied to tissue where a precise and sensitive localization of intracellular monoamines both in neuronal and non-neuronal tissue is possible. The new technique has proved extremely useful for sensitive and detailed neuroanatomical studies on catecholamine neurons in the CNS.

Ekfelt T, Fuxe K, and Goldstein M (Department of Histology Karolinska Institute 104 01 Stockholm Sweden and Department of Psychiatry New York Medical Center New York U.S.A.): IMMUNOHISTOCHEMICAL VISUALIZATION OF CATECHOLAMINE SYNTHESIZING ENZYMES 60

Three catecholamine synthesizing enzymes: dopa decarboxylase (DDC), dopamine- β -hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT) were purified from bovine adrenal medulla and antibodies to these enzymes (anti-DDC, anti-DBH and anti-PNMT) were produced in rabbits. The antibodies were used in immunohistochemical studies (indirect technique) on the adrenal medulla and the peripheral and central nervous system (CNS).

DDC and DBH were present in all gland cells of the adrenal medulla whereas PNMT could be demonstrated only in a peculiar population supporting the concept of two cell types in the adrenal medulla storing no adrenaline (NA) and adrenaline (A) respectively. Objective and subjective estimations of enzyme levels were performed on the adrenals after various experimental procedures. It was found e.g. that ACTH increased the PNMT level of hypophysectomized rats.

In the CNS all parts of the monoamine neurons could be identified with this technique. Furthermore, dopamine (DA) (containing DDC but not DBH) and NA (containing DBH) neurons could be separated. 5-Hydroxytryptamine (5-HT) neurons could also be visualized with anti-DDC suggesting that DA and 5-HT neurons contain an antigenically undistinguishable carboxylase. In addition, catecholamine systems described on the basis of the Falck-Hillarp technique (DDC-positive cells) could be identified in certain hypothalamic areas.

61 Ljungdahl, Å and Håkfelt, T (Department of Histology Karolinska Institutet, 104 01 Stockholm Sweden): AUTORADIOGRAPHIC DEMONSTRATION OF ^3H -GABA AND ^3H -GLYCINE UPTAKE IN SPINAL CORD

γ -aminobutyric acid (GABA) and glycine have been postulated to mediate pre- and postsynaptic inhibition in the spinal cord but the localization of the presumed GABA and glycine neurons is not known. In the present study light and electron microscopical autoradiography has been used to locate the uptake of ^3H -GABA and ^3H -glycine in rat and cat spinal cord. Both *in vivo* injections of the isotopes directly into the spinal cord and *in vitro* incubations of tissue slices were used.

^3H -glycine is accumulated in nerve terminals often characterized by their content of flat vesicles in myelinated axons in glial cells and in certain neuronal cell bodies. These cell bodies are mainly located in the anterior horn.

^3H -GABA is accumulated in nerve terminals often making axo-axonic contacts with non-labeled boutons. This is of interest in view of the proposed role of GABA in presynaptic inhibition. ^3H -GABA is also accumulated in glial cell and neuronal cell bodies. These neurons have a more dorsal location than ^3H -glycine accumulating cells.

In contrast ^3H leucine is taken up by all cells with no accumulation in nerve terminals.

Thus it is possible to map specific neuronal systems in the spinal cord by autoradiography with ^3H -GABA and ^3H -glycine.

ERE!

62 Seiger, Å and Olson, L (Dept of Histology Karolinska Institutet Stockholm Sweden): HISTOCHEMICAL DEMONSTRATION AND MAPPING OF CENTRAL MONOAMINE NEURONS IN THE RAT: THE PRENATAL ONTOGENY

The development of all monoamine-containing neuron systems in the rat brain throughout the prenatal period of development has been analysed by Falck-Hillarp fluorescence histochemistry (Olson and Seiger *Z Anat Entw Gesch* 1972 301; Seiger and Olson *Z Anat Entw-Gesch* 1973 in press). It has been possible by serial sagittal, horizontal and transverse sections through whole brains from 10 to 22 day old fetuses to map the development of

11 monoamine cell groups known in the adult animal: a large number of axon bundles and their formation of terminal plexuses in different receptor areas. Only fetuses from MAO-inhibited mothers were analyzed because of the marked increase of intraneuronal amine levels by this pretreatment.

The first monoamine cell group to appear is the 5-HT group in rostral pons and caudal mesencephalon on the 12th day of gestation (crown rump length CRL 8 mm). The mesencephalic DA cell complex appears a little later (CRL 9 mm) and the pontic NA cell complex can be seen at the 14th day of gestation (CRL 11-12 mm). The common origin of different monoamine cell groups described in adult animal has been determined. Thus A1-A3, A4, A7, A8, A10, B1-B3, B4-B9 respectively (nomenclature according to Dahlström and Fuxe 1964) have common origins. New neuroanatomical data has been obtained mainly from 5-HT axon projections not previously described. Ascending bundles are seen from groups B6, B7, B8, B9 and probably from B3 and B5. Descending bundles are seen from groups B1, B2, B3, B6 and probably from B5, B7, B8 and B9. It is concluded that all the monoamine cell groups present in the adult animal appear and at least partly develop prenatally.

DEVELOPMENTAL CHANGES OF THE NEUROTOXIC ACTION OF 6 HY 63
DROXYDOPAMINE (6-OH DA) ON CENTRAL NORADRENALINE (NA) NEU-
RONS Charlotte Sachs and Gösta Jonsson Dept. of Histology Karolinska
Institutet Stockholm.

The neurotoxic substance 6-OH DA does not readily pass the blood brain barrier (BBB). However systemic injection of 6-OH DA to newborn rats produces a selective and permanent degeneration of a considerable number of NA nerve terminals in the brain (60% reduction of ^3H NA uptake in cerebral cortex and spinal cord; 40% reduction in the hypothalamus) thus indicating that 6-OH DA can pass BBB in the newborn stage. In order to study the development of BBB to 6-OH DA rats received 6-OH DA (3x100 mg/kg s.c.) at various periods of time after birth. The neurotoxic action of 6-OH DA was evaluated by measuring the in vivo uptake of ^3H NA in various brain regions when the rats had reached the adult stage. There was a rapid development of a barrier and the ^3H NA uptake was completely restituted in the cerebral cortex when 6-OH DA was given on day 9 postnatally while in the hypothalamus on day 5. No effective barrier seemed to develop in the spinal cord. 6-OH DA per se had no apparent effect on the outgrowth of the NA nerves that did not undergo degeneration. However analysis of ^3H NA uptake in cerebral cortex shortly after the 6-OH DA injection in rats older than 9 days showed a small (about 25%) transient reduction in ^3H NA uptake in rats younger than 28 days while in rats older than 28 days this effect was permanent. These results show that the BBB to the neurotoxic action of 6-OH DA although not completely protective develops shortly after birth. The NA neurons possess up to about 28 days postnatally a certain regenerative capacity which later disappears.

Olson, L. and Seiger, I. (Dept. of Histology Karolinska Institutet Stockholm, Sweden): BRAIN TISSUE TRANSPLANTED TO THE ANTERIOR CHAMBER OF THE EYE: MONOAMINE NEURONS REINNERVATING THE RAT IRLS 64

Transplantations to the anterior chamber has previously been used to study growth of sympathetic neurons (Olson and Malmfors Acta physiologica 1970 Suppl. 348). The prenatal ontogeny of the CNS monoamine neurons has recently been described (Olson and Seiger I Anat. Entw. Gesch. 1972 137-301; Seiger and Olson I Anat. Entw. Gesch. 1973 in press). Knowing the development of the noradrenaline (NA) dopamine (DA) and 5-hydroxytryptamine (5-HT) neurons it is possible to obtain by microsection small pieces of immature brain containing the various monoamine neurons. Following homologous transplantation to sympathectomized eye of adult rat the transplants readily survive, become vascularized and mature intraocularly. The method permits in vivo observations of the condition of the transplants.

The transplant and its development was analysed by Fick-Hill type fluorescence histochemical try (Olson and Seiger I Zellf. 1972 135-175). All three types of monoamine neurons survived in the transplant and innervated it in at least partly homotypical patterns. Furthermore the intralaminar monoamine neurons were all able to reinnervate the sympathectomized host iris forming plexus as similar to the normally present sympathetic ground plexus. Catecholamine and 5-HT fibers had the same pathway on the iris when transplanted together. From this and similarly designed experiment it is concluded that all the three types of monoamine neurons are able to innervate denervated sympathetic receptor. The pattern of reinnervation and morphology of the outgrowing fibers is determined by the acceptor tissue.

- 65 Tuomisto, J (Department of Pharmacology University of Helsinki Finland)
DOPAMINE UPTAKE IN STRIATAL SYNAPTOSOMES CONFORMATIONAL SELECTIVITY

Noradrenaline (NA) and its sterically rigid analogs were used as inhibitors of dopamine (DA) uptake in striatal synaptosomes. NA was rendered conformationally rigid by incorporating its active groups into the trans-decalin structure. Four isomers were used in three of them the relation of the catechol ring and the amino function was gauche. In one it was anti. Crude synaptosomal fractions were prepared according to Snyder and Coyle (J Pharmacol exp Ther 165:78, 1969). The synaptosomes were incubated with 10 μ M tritiated DA and with or without an inhibitor for 5 min in Krebs-Henseleit bicarbonate buffer. One of the gauche isomers 2(a) amino-3(e)-3,4-dihydroxyphenyl-3 trans-decalol had 50% of the potency of NA as DA uptake inhibitor. Its IC_{50} (concentration inhibiting 50% of uptake) was 2.4×10^{-6} M. The inhibition was competitive. The anti isomer was 22 times weaker as an inhibitor (IC_{50} 5.2×10^{-5} M).

These results suggest that the DA transport mechanism in striatal nerve terminals is conformationally selective and the conformer having the highest affinity for the transport is gauche. It is noteworthy that according to molecular orbital calculations (Kier and Truitt, J Pharmacol exp Ther 174:94, 1970) the preferred conformation of DA is gauche but that of NA is anti. Thus the transport mechanism in the striatum would favour DA for NA since only the former is predominantly in the gauche form.

- 66 Squires, Richard (Research Laboratory A/S Ferrosan, Søborg, Denmark): EFFECTS OF SOME PSYCHOTROPIC DRUGS ON THE SIMULTANEOUS UPTAKE OF 5-HT AND NORADRENALINE BY SYNAPTOSOMES

Synaptosomes are prepared from rat forebrain by gentle homogenization in 0.32M sucrose containing 1mM Nialamide. Unbroken cells and debris are removed by low speed centrifugation and the whole supernatant used as a source of synaptosomes. Synaptosomes are mixed in ice bath with modified oxygenated Krebs-Ringer test substance plus radioactive (C^{14}) 5-HT (0.05 μ M) and (H^3) noradrenaline (25 μ M) (final conc.). The mixture is transferred from ice bath to 37°C for 20 min, then returned to ice bath to stop uptake. Synaptosomes are then collected on Millipore filters, washed with cold 0.9% NaCl and counted in Instagel in a liquid scintillation counter. 5-HT uptake was inhibited 60% by chlorimipramine (14 μ g/ml), chlorpheniramine (94 μ g/ml), imipramine (138 μ g/ml), amitriptyline (168 μ g/ml), protriptyline (566 μ g/ml) and desmethylinipramine (623 μ g/ml). The concentrations of these substances required to inhibit the uptake of NA 50% was generally much higher: chlorpheniramine (0.22 μ g/ml), protriptyline (0.85 μ g/ml), imipramine (1.7 μ g/ml), amitriptyline (1.4 μ g/ml), desmethylinipramine (1.8 μ g/ml), imipramine (2.0 μ g/ml). A preliminary comparison of 5-HT, NA and dopamine uptake inhibition in synaptosomes from different brain regions suggests that under the conditions described above, 5-HT is taken up almost entirely by specific 5-HT synaptosomes while NA maybe taken up mainly by DA synaptosomes in all regions of the brain examined.

Tetrahydroharmane alkaloids have an indolylethylamine moiety in their structure and it is feasible that these compounds may be formed in vivo as condensation products of 5-hydroxytryptamine (5HT) and acetaldehyde. Effects of these compounds were studied on monoamine uptake in rat brain stem synaptosomes. Crude synaptosomes were prepared according to Snyder and Coyle (J Pharmacol exp Ther 1969 163 78) and incubated for 5 min with radioactive noreadrenaline (NA), dopamine (DA) or 5HT in Krebs-Henseleit bicarbonate buffer. 1,2,3,4-tetrahydroharmane (TH) and 6-hydroxy-1,2,3,4-tetrahydroharmane (6HTH) were equipotent as catecholamine uptake inhibitor and the ID₅₀ (concentration inhibiting 50% of uptake) was $2.5 \times 10^{-5} M$ for NA and $7 \times 10^{-5} M$ for DA. As 5HT uptake inhibitor 6HTH was slightly more active (ID₅₀ $4 \times 10^{-6} M$) than TH (ID₅₀ $8 \times 10^{-6} M$). In all instances these compounds were more potent than salutaridinol which is the corresponding condensation product of DA. However, also the potency of TH and 6HTH was clearly lower than that of the parent amines 5HT and tryptamine.

It is concluded that tetrahydroharmane derivatives have affinity to the amine transport mechanisms in the synapse, but the affinity is lower than that of the primary indolylethylamines.

Winblad, B (Research Institute of National Defence, Sundbyberg, Sweden): 68
CHOROID PLEXUS UPTAKE OF ACETYLCHOLINE:

Acetylcholine (ACh) is accumulated by rabbit choroid plexus in vitro by active transport. The transport system seems to be identical with that for choline (Ch) and has a high capacity. Calculated maximal speed of uptake varied between 10 and 60 $\mu moles \times min^{-1} \times kg^{-1}$ wet weight. The choroid plexus contained a significant cholinesterase activity (ChE) which seemed to be localized mainly intravascularly. ACh was not metabolized by the tissue i.e. intravascular concentrations of ACh must have been low. The cholinesterase inhibitor (ChEI) physostigmine inhibited ACh and Ch uptake while the two ChEIs scopolamine and sarin stimulated the uptake. The relative stimulation of the ACh uptake by scopolamine increased with increasing ACh concentration in the incubation medium. Scopolamine enhanced the release of ACh from tissue preincubated for 15 min at $10^{-3} M$ but not from tissue preincubated at $10^{-4} M$ ACh in preliminary experiments. The results will be discussed in relation to ACh and Ch transport in brain slices. Further possible localizations of the transport mechanism will be suggested.

- 69 Nordberg, A and Sundvall, A (Dept of Pharmaceutical Pharmacology Biomedical Center Box 573 S-751 23 Uppsala Sweden): EFFECT OF PENTOBARBITAL ON THE TURNOVER OF ACETYLCHOLINE IN DISCRETE REGIONS OF MOUSE BRAIN IN VIVO

It has recently been shown that although barbiturate anaesthesia produces increased steady state levels of acetylcholine (ACh) in the brain it markedly decreases the turnover of the transmitter (Schubert J Sparf B and Sundvall A J Neurochem. 16 695 (1969)). Since cholinergic neurons are not evenly distributed it was considered of interest to study the effect of pentobarbital on steady state concentration and turnover in different regions of the mouse brain.

The animals were killed by dislocation of the spine and the brains were rapidly dissected on ice into six welldefined parts (cerebellum medulla oblongata midbrain striatum hippocampus and cortex). The tissue was homogenized in TCA and the ACh in the extract was estimated by bioassay using a strip of the dorsal muscle of the leech in a microbath. The results are summarized in the table. As seen there were marked differences regarding regional ACh values both in normal and pentobarbital treated animals.

	ACh in nanomoles/g brain					
	Cerebellum	Medulla obl	Midbrain	Striatum	Hippocampus	Cortex
Control	3.73	22.77	18.11	36.85	17.40	11.14
Increase	n.s.	n.s.	57	n.s.	79	163

+ 2/5 samples 125% increase

Turnover was studied by measuring the rate of formation of radioactive ACh in different parts of the brain following intravenous injection of radioactive choline (Ch). A marked decrease was demonstrated in hippocampus and cortex following an anaesthetic dose of pentobarbital.

- 70 Gudrun Paulsow and Lennart Paulsow (Departments of Pharmacology, B.M.C., Box 573 S-751 23 Uppsala and Apotekens Centrallab., Box 3045, S-171 03 Solna 3 Sweden): THEOPHYLLINE INCREASED SENSITIVITY TO
 100% AMP STIMULATION AND REGIONAL TURNOVER OF RAT BRAIN 5-HT
 HIAA NORADRENALINE AND DOPAMINE

We have previously reported (Acta pharmacol. et toxicol. 32, 22, 1973) that theophylline and caffeine are able to decrease the normal pain threshold in a dose-dependent manner. These methylxanthines are known to increase the level of cyclic AMP and considerable evidence suggests that cyclic AMP may participate in the synaptic transmission. We have now looked more closely upon the relationship between the increased sensitivity to painful stimulation after administration of theophylline and the regional turnover of rat brain indole- and catecholamines.

The rats were killed 30 min. after injection of 100 mg/kg theophylline i.p. and the brain was dissected into three parts: cortex, forebrain and brain stem. Responses to noxious stimulation were divided into motor response, vocalization and vocalization after discharge.

Theophylline was found to be able to decrease the turnover of dopamine in cortical and forebrain regions while that of noradrenaline was unaffected. An increased level of 5-HIAA was found in the brain after theophylline and this increase was more pronounced after probenecid treatment. In cortical regions the level of 5-HIAA was decreased. Endogenous serotonin was unaffected in all regions studied. Probenecid per se antagonized the theophylline decreased threshold for vocalization after discharge and the cortical decreased 5-HIAA.

Wiesel, F A., Fri, C G. and Sedvall, G. (Dept of Pharmacology 71
Karolinska Institutet Stockholm Sweden) EFFECT OF PARGYLINE
ON HOMOVANILLIC ACID IN RAT STRIATUM AS MEASURED BY MASS FRAG
MENTOGRAPHY

Earlier methods have lacked enough sensitivity for determin-
ation of homovanillic acid (HVA) turnover in rat striatum using
a monoaminoxidase inhibitor. This has now been possible since
a mass fragmentographic method has been developed for measuring
HVA in the picomole range in brain tissue (Sjöquist et al
J Neurochem 1973 in press)

Male Sprague Dawley rats were injected with pargyline 75 mg
per kg i.v. and killed at different time intervals. Striata
were homogenized in 0.1 M formic acid and 50 μ M ascorbic acid.
Each sample was divided for determination of HVA and dopamine
(DA) (Koslow et al Science 1972 176 177). The steady state
levels of HVA and DA were found to be 4 and 57 nmole/g respec-
tively. After pargyline treatment the HVA levels disappeared
following an exponential curve. The half life was calculated to
12 minutes. HVA turnover was estimated to be at least 14 nmole
per g per h. This is in agreement with data found by Spano and
Neff (1972) using probenecid as a tool for calculating HVA
turnover in guinea pig striatum. The DA levels increased about
40% within 80 minutes.

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Lindqvist M., Carlsson A. and Kehr W. (Department of Pharmacology 72
University of Göteborg Sweden): INFLUENCE OF ANAESTHETICS ON TYRO-
SINE AND TRYPTOPHAN HYDROXYLATION IN RAT BRAIN IN VIVO

Rats were exposed to ether during 15-60 min and NSD 1015 (3-hydroxybenzyl
hydroxylase HCl) an inhibitor of aromatic amino acid decarboxylase 100 mg/kg
i.p. was given either during or at various times after ether anaesthesia. Ether
caused a rapid acceleration of dopamine and 5-HTP accumulation after decarboxylase
inhibition during the initial 15-30 min of anaesthesia, while the accumulation was
decreased below control values in the period after ether. These changes in tyro-
sine hydroxylation were most pronounced in the limbic DA-1ch system and especial-
ly in the corpus striatum, whereas the changes in tryptophan hydroxylation were
less marked and about similar in all brain parts.

Similar changes in tyrosine hydroxylation were also observed after ethanol treat-
ment (0.5-4 g/kg). After NSD 1015 the dopamine accumulation in the brain was
accelerated by ethanol both in the corpus striatum and in the hemispheres. How-
ever, no concomitant effect on 5-HTP levels was found.

Narbutool anaesthesia (40 mg/kg) had no marked influence on monoamine
synthesis in the brain.

Ether and ethanol seems to have similar actions on monoamine synthesis in cate-
cholamine neurons, but only ether has in addition an effect on 5-HT neurons.

- 73 Schubert J Fyrö B and Sedvall M (Dept of Psychiatry (St Göran's Hospital) and Pharmacology Karolinska Institutet Stockholm Sweden) SYNTHESIS OF MONOAMINES FORMED FROM LABELLED PRECURSORS IN RAT BRAIN DURING LITHIUM TREATMENT

Chronic treatments with lithium were performed with two doses of LiCl added to the food resulting in lithium levels in serum of 0.4 and 0.7 meq/l respectively. At the lower dose monoamine synthesis in brain was not affected. With the higher dose accumulation of labelled 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid in brain during i.v. infusion of ^3H tryptophan was increased. This effect was present in the spinal cord following acute spinalization, thus occurring independently of nerve impulse activity. Disappearance from brain of labelled 5-HT formed from ^3H tryptophan was reduced in lithium-treated animals indicating that lithium diminishes release of 5-HT from its storage sites. Brain tryptophan was increased after lithium, whereas levels of free and total tryptophan in serum as well as its catabolism reflected by liver tryptophan pyrrolase activity were unaltered. Accumulation and disappearance of labelled dopamine and noradrenaline formed from ^{14}C tyrosine in brain were not changed significantly by any of the lithium diets.

The results suggest that chronic treatment of rats with lithium within the low dose range recommended for therapy in man affects 5-HT but not catecholamine synthesis in brain.

Supported by grants from the Swedish Medical Research Council (14X 2381 and 21X 2291) and Karolinska Institutet.

- 74 Antorp O & S C Sørensen (Institute of Medical Physiology Dept A University of Copenhagen Denmark): ONTOGENESIS OF THE BRAIN BARRIER IN RATS:

The exchange of hydrophilic substances between blood and interstitial fluid is impeded in the brain compared to other tissues. Furthermore the so-called blood-brain barrier is able to maintain concentration differences for various ions by means of active transport mechanisms. We have in rats studied the development of the barrier to macromolecules and of the transport processes for K^+ , Na^+ and Cl^- by measuring the concentration of total protein, K^+ , Na^+ and Cl^- in cisternal cerebrospinal fluid (csf) and plasma from newborn rats and from rats of various ages. The concentration ratio csf/plasma (mean \pm SD) for proteins was 0.091 ± 0.007 at birth and did not reach the adult value of 0.0053 ± 0.0001 until 24 days after birth. In contrast the concentration ratios csf/plasma H_2O for K^+ , Na^+ and Cl^- were at the time of birth not significantly different from the values found in adult rats i.e. 0.63 ± 0.07 , 0.99 ± 0.02 and 1.15 ± 0.03 respectively. Thus the ability to maintain concentration differences for small ions develops before the blood-brain barrier becomes tight to macromolecules. This may indicate that the tightness to macromolecules and the active transport of small ions may be attributed to different cell layers e.g. endothelium and perivascular astrocytes respectively. It must however also indicate a large capacity of transport systems of the blood-brain barrier enabling maintenance of concentration differences despite a large intercellular leak.

Lund-Andersen H. & M. Møller (Department of Biochemistry A and Anatomy Department A University of Copenhagen Denmark): **EXTRACELLULAR AND INTRACELLULAR DISTRIBUTION OF INULIN IN RAT BRAIN-CORTEX SLICES**

Inulin space is commonly used to indicate the magnitude of the extracellular space in brain-cortex slices. It has however been shown (Lund-Andersen & Møller, Biochem. Soc. Trans. 1, 1973, 123) by examination of the wash out course of ^{14}C -labelled inulin that 2/3 of the amount taken up during 60 min of incubation is localized in the extracellular space whereas 1/3 is intracellularly located. The 'true' extracellular space can be calculated to 29 μl per 100 mg initial wet weight. A large extracellular space compared with uncubated slices is correspondingly observed in electron micrographs. The amount of extracellularly located inulin is reduced by 50 per cent when cellular swelling is evoked by 35 mM KCl; electron micrographs confirm the reduction of the extracellular space and furthermore shows that the KCl evoked swelling is a glial cell phenomenon (Zadumaisky et al. Exp. Neurol. 8, 1963, 290).

It is concluded 1) that the quantitative determination of the magnitude of the intra- and extracellular compartments by aid of inulin diffusion kinetics is in agreement with the qualitative electron microscopically findings. 2) That the potassium-induced swelling is a glial cell phenomenon. 3) That intracellular penetration of inulin in brain tissue may be a physiological phenomenon or may indicate *in vitro* cell membrane damage. 4) and that the traditionally defined inulin space is not a measure of the extracellular space in brain-cortex slices.

Aarseth P. and B. A. Wessler (Institute of Physiology University of Oslo Norway): **INTERSTITIAL WATER CONTENT IN ISOLATED RABBIT LUNGS AT DIFFERENT LEVELS OF CAPILLARY PRESSURE**

It has been questioned whether the Starling concept for transcapillary fluid balance applies to the pulmonary vascular bed. The existence of a small volume of interstitial fluid with a large protein-concentration could explain how changes in hydrostatic capillary pressure is balanced without much extra accumulation of perivascular fluid. We wanted to test whether there is only such a small augmentation of extravascular fluid after elevation of lung capillary pressure. Isolated rabbit lungs were perfused with plasma and their weight continuously followed. Extravascular water content and plasma volume were calculated in removed lobes with the use of labelled plasma albumin and by measurement of wet weight/dry weight ratios. At stable vascular pressures the extravascular water content of the upper lobes differed by less than 3%. In test experiments the left atrial pressure was elevated by 10 mm Hg. One upper lobe was removed before the other 8 min after this pressure elevation. At this time the preparations were approaching weight stability indicating that transcapillary fluid balance was nearly achieved. In the lobes exposed to such elevated vascular pressures we could either record no changes in extravascular water content or an augmentation of 10% or less on average. The extravascular fluid compartment involved in transcapillary fluid balance in the lungs is thus apparently very small.

- 77 Parving H -H₁ Rossgaard M Nielsen S E & Lassen N A (Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark): INCREASED TRANSCAPILLARY ESCAPE RATE OF PLASMA PROTEINS DURING ALBUMIN AND DEXTRAN LOADING IN MAN

The transcapillary escape rate (TER) of a plasma protein is defined as that fraction of the intravascular mass of the protein which passes to the extravascular space per unit time. TER of plasma albumin was determined as the initial disappearance rate of 1 v injected radioiodinated albumin in 4 human subjects before and after 1 v loading with 95 g albumin. Albumin loading significantly increased TER from 4.6 (S.D. 1.08) to 8.1 (S.D. 2.0) %/hr. The consequence was a net transfer of 11% of the plasma albumin mass to the extravascular space during the first 5 hrs after the loading. There was also a net transfer of other proteins as reflected by a reduction of the masses of intravascular IgG and IgM of 9 and 7 % respectively. Similar results were obtained in 4 other subjects loaded with 900 ml Dextran (Mw 70 000).

Our results can be explained by increased microvascular permeability (the stretched pore phenomenon) and/or by increased filtration due to the venous pressure elevation caused by the loading.

- 78 Poulsen, H L (Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark): SUBCUTANEOUS INTERSTITIAL ALBUMIN CONCENTRATION IN LONG-TERM DIABETES MELLITUS

By a wick method human interstitial fluid was collected from the subcutaneous tissue of the forearm. 12 normal men had an average interstitial fluid albumin concentration of 2.66 g/100 ml (S.D. 0.30) and a ratio between interstitial albumin concentration and serum albumin concentration of 0.63 (S.D. 0.09). 8 male juvenile long-term diabetics matched for age, had the corresponding values of 1.46 g/100 ml (S.D. 0.47) and 0.38 (S.D. 0.12) respectively ($p < 0.001$). In 3 male juvenile short-term diabetics normal values were found.

In view of other reports pointing to increased microvascular albumin permeability in long-term diabetes the results suggest a much enhanced net water flux (lymph formation) in the tissue studied.

CONCENTRATION IN EXPERIMENTAL HYPOPROTEINEMIA

Absence of edema in hypoalbuminemia suggests compensatory changes in the transcapillary Starling forces for instance
1) increased interstitial hydrostatic pressure (P_i) or
2) a greater reduction of albumin concentration in interstitial fluid (A_i) than in plasma (A_p)

These possibilities were investigated in rats during development of aminonucleoside nephrosis. P_i was measured with a modified Scholander wick method with the wick contained in a 20 gauge cannula provided with a sidehole. A_i was determined in wicks after 1 hour subcutaneous implantation (Aukland & Fadnes Acta physiol scand 1972 84 26A). Daily injections of 5-6 mg Puromycin aminonucleoside induced proteinuria and progressive hypoalbuminemia after 4-5 days. P_i ranged from 0 to -2 cm H₂O both under control conditions and in hypoalbuminemia. Control A_p and A_i averaged 3.5 and 2.0 g/100 ml. At the 5th day A_p was reduced by about 1 g/100 ml and an even greater reduction was observed in A_i when A_p was further reduced to less than 2 g/100 ml. A_i fell to less than 0.3 g/100 ml.

It is suggested that the disproportional reduction of A_i is caused by increased capillary filtration and lymph flow and in turn acts to limit edema formation. P_i does not seem to be of importance for preventing subcutaneous edema.

Auranes I and J Vaage (Institute of Physiology University of Oslo Norway) CHANGES IN PERIPHERAL LYMPH DURING INTRA-VASCULAR AGGREGATION OF BLOOD PLATELETS

Several investigators claim that blood platelets may yield components affecting vascular permeability after various types of in vitro treatment. We wanted to study the effects on microvessels of intravascular aggregation of blood platelets.

The number of erythrocytes was estimated in peripheral lymph collected from a major lymph vessel in the hind limb of cats. Blood pressure (BP), hematocrit (hct) and the number of circulating blood platelets were followed. Intravascular platelet aggregation was induced by infusion into the abdominal aorta over one hour of a collagen suspension.

During this infusion the BP decreased moderately. The level of circulating blood platelets fell to about 30 per cent of the initial value. The number of erythrocytes in the lymph normally less than 1000 per mm³ increased to a peak value of about 10000 per mm³. Hct rose by about 25 per cent and lymph flow increased considerably.

When the collagen infusion was ended hct and lymph flow rapidly decreased. The number of erythrocytes in the lymph did gradually return to a normal value during the next 3 hours.

We conclude that intravascular aggregation of blood platelets can induce acute changes in the wall characteristics of the microvessels. This change may be mediated through substances released from the blood platelets.

Paaske W P & Levin Nielsen (Institute of Medical Physiology B University of Copenhagen Denmark & Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark): VASCULAR PERMEABILITY IN ADIPOSE TISSUE MEASURED BY THE SINGLE INJECTION EXTERNAL REGISTRATION METHOD

Measurement of capillary permeability in adipose tissue with available methods has proved difficult due to the low perfusion of this tissue in vivo. The single injection external registration method using bolus injection of gamma-emitting radio-nucleides (Sejrsen P. In Capillary Permeability Munksgaard, Copenhagen 1970) was therefore applied to adipose tissue.

The present study describes the theoretical basis of the method and suggests a technique of fast on-line computation of capillary extraction and capillary diffusion capacity combined with graphical plotting of the externally recorded triexponentially fitted curve function.

After bolus injection of ^{51}Cr -EDTA into the artery of the isolated inguinal fat pad in slightly fasting female rabbits the capillary extraction and capillary diffusion capacity were found to be essentially similar to values reported from experiments on skeletal muscle. It is concluded that the single injection external registration method is of considerable value for determination of capillary permeability in adipose tissue.

Aarseth, P., G. Bø and H. Piene (Institute of Physiology University of Oslo): PLASMA HYPEROSMOLARITY AND PULMONARY FLUID COMPARTMENTS

Plasma hyperosmolality has a marked dilatory effect on muscle resistance vessels and some dilatory effect also on pulmonary capacitance vessels. In contrast to this systemic capacitance vessels are not much influenced by hyperosmolality. The pulmonary capacitance vessels of isolated lungs appeared to constrict when the plasma osmolality was raised (Bø Hauge and Nicolaysen Acta physiol scand 1971 82 375).

In the present investigation we have estimated the blood- and extravascular water volumes in the lungs of 7 intact cats during infusions of hyperosmotic solutions (3M NaCl 3M and 5.9M glucose). These volumes were estimated by measuring plasma and erythrocyte radioactivity in rapidly snared-off lung lobes. After a mean increase of 18% in the plasma osmolality the blood content of a lobe No 2 was increased by 24% relative to that of a lobe No 1 removed before infusion. This increase is however not significantly greater than what was found in a control group of 3 cats where the 2 lobes were removed without any intervening infusions. The extravascular water volume during hyperosmolality fell by 21% which is significantly different from a minor change seen in the control group.

It is concluded that increase in plasma osmolality has no detectable effect on the pulmonary capacitance vessels whereas it will markedly reduce the extravascular lung water volume.

Nilsson, G. (Department of Pharmacology, Karolinska Institute, 83
Stockholm, Sweden): EFFECT OF ACID IN THE DUODENAL BULB ON FASTING
GASTRIC AND GASTRIN SECRETION

Bulbar acidification inhibits gastric acid secretion in response to
test meal sham feeding, insulin hypoglycemia, exogenous gastrin but
not to histamine. The present study was undertaken to determine if
bulbar acidification inhibits fasting gastric acid secretion and basal
plasma levels of gastrin.

Dogs were provided with fully innervated pouches of the fundic
stomach and of the proximal portion of the duodenum corresponding
the duodenal bulb. Acid perfusion of the bulbar pouches abolished
the spontaneous acid secretion from the Pavlov pouches but did not
influence the plasma concentration of gastrin.

The results may mean that lowering of the intrabulbar pH plays a
role in suppressing gastric acid secretion appearing between meals.
Such inhibition is not induced by reduction of plasma concentra-
tions of gastrin. Instead it may be due to the hypothetical hor-
mone bulbogastrone acting at the acid secreting glands.

Sjödin, I., and Nilsson, G. (Department of Pharmacology, Karolinska 84
Institutet, Stockholm, Sweden): GASTRIC ACID SECRETION AND PLASMA
GASTRIN LEVELS DURING URECHOLINE INFUSION IN THE DOG

Cholinergic stimulation of gastric acid secretion may be induced by
different means such as sham feeding or hypoglycemic agents. Such
cholinergic activation of acid secretion is thought to be due to both
direct excitation of the parietal cells and release of antral gastrin.
In the present study, the effects of a stable choline ester (Ure-
choline[®]) on gastric acid secretion and plasma gastrin levels were
investigated.

Each of 4 dogs was provided with a vagally denervated fundic pouch
and a draining cannula in the main stomach. A continuous intravenous
infusion of 50 µg per kg-hr of Urecholine did not influence plasma
gastrin concentration as determined by radioimmunoassay. Administra-
tion of 100 µg per kg-hr significantly raised the plasma gastrin
level in only 3 out of 10 experiments. However, in all experiments,
Urecholine did cause marked acid secretion from the gastric pouches.

The results show that cholinergic stimulation by Urecholine may
evolve gastric acid secretion without influencing the plasma level of
gastrin. This suggests that basal plasma levels of gastrin suffice
to potentiate the direct effect of Urecholine on the parietal cells.

85 Berglinh T, K, J Öbrink and M Waller (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) VASOPRESSIN AS INHIBITOR OF ACID SECRETION IN THE ISOLATED GASTRIC MUCOSA

Isolated gastric mucosae from *Rana Temporaria* were mounted as a membrane in a two chamber system. The nutrient side chamber contained a buffered frog-Ringer's solution gassed with 5% CO₂ and 95% O₂. The secretory side faced an unbuffered salt solution gassed with pure O₂. The mucosae were not stimulated unless they showed a complete secretory rest. Then histamine to a final concentration of 10⁻⁷ mol/lit was given. Consequently there were two sets of preparations: 1) those with a spontaneous secretion and 2) those with a histamine induced secretion.

Lysin-vasopressin was added to the nutrient side to a final concentration of approximately 10⁻⁸ mol/lit. The effect was a striking inhibition of the spontaneously secreting stomachs but no effect at all of the histamine stimulated ones.

86 Frenning B (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) NET TRANSPORT OF SODIUM IONS INTO THE RESTING STOMACH

170 mM HCl was instilled into resting cat stomachs mounted *in vivo* as the of a cylindrical perspex chamber (Frenning B Uppsala J Med Sci 1972 Suppl. 13). A net movement of sodium ions into the instillate without any change of its volume was observed. This result suggests a diffusional transport of sodium ions into the stomach (Teorell T J gen Physiol 1939 23: 263). However other authors believe that sodium ions enter the stomach merely as constituent of a diluting and/or neutralizing secretion (for review see Hunt J N and Wan B Handbook of Physiology sect 6, II p 781, 1967).

To examine further the net movement of sodium ions into the resting stomach 170 mM HCl was instilled into the chamber and physiological saline or 170 mM HCl infused into the instillate at different constant rates. At the end of the experiments the total amount of sodium ions in the gastric contents was determined and the amount infused subtracted. When physiological saline was infused it was found that the amount of sodium ions that moved into the stomach (corrected for that infused) was lower the higher the infusion rate. When 170 mM HCl was infused the net movement of sodium ions into the stomach remained at the control level.

The results suggest that a considerable part of the net transport of sodium ions into the stomach occurred by diffusion.

Rasmussen S (Department of Pharmacology Royal Danish School of Pharmacy DK-2100 Copenhagen O Denmark): THE EFFECT OF PHOSPHATE ON CALCIUM TRANSPORT IN ISOLATED RAT SMALL INTESTINE IN VITRO

Unidirectional Ca-fluxes were measured in isolated rat distal jejunum in a modified Ussing-apparatus. At Ca-concentration of 0.25 mM the following fluxes as nMol/cm²h[±]s.e.m. were measured:

mM PO ₄	J _{ms}	J _{ss}	J _{net}	
0	5.9 ± 2 (5) ^x	5.1 ± 3 (5)	0.8	P > 0.1
1	7.5 ± 2 (6)	4.9 ± 2 (6)	2.6	P < 0.01
2.5	7.0 ± 4 (5)	4.6 ± 5 (5)	2.4	P < 0.01
5	6.4 ± 2 (5)	4.4 ± 2 (5)	2.0	P < 0.01

J_{ms}: flux mucosa to serosa J_{ss}: flux serosa to mucosa J_{net}: net flux x). No. of experiments. The results are in agreement with those by Helbock et al (Biochim Biophys Acta 126 1966 81) who found net Ca-transport only in the presence of phosphate but in contrast to other authors e.g. Wasserman & Taylor (Mineral Metabolism Vol III C L Comar & F Bronner Ed Academic Press 1969 p 321) who did not find phosphate necessary for the net-transport.

Damgaard Nielsen G & S Rasmussen (Department of Pharmacology Royal Danish School of Pharmacy DK-2100 Copenhagen O Denmark): EFFECT OF VITAMIN D ON THE CALCIUM INFLUX ACROSS THE BRUSH BORDER MEMBRANE OF ISOLATED RAT SMALL INTESTINE IN VITRO

Influx of Ca at 20 mM across the brush border membrane of the rat distal jejunum was measured by the method by Schultz et al (J Gen Physiol 50 1967 1241) modified for rat intestines. Influxes measured in rats bred and maintained on a vitamin-D free diet (all kindly supplied by Statens Levnedsmiddelinstitut Copenhagen) were found to be 2.12 ± 0.11 μMol/cm²h. No. of experiments: 7. Vitamin-D deficient rats kept on a rachitogenic diet for three weeks show influxes of 1.86 ± 0.15 (7) μMol/cm²h. After administration of cholcalciferol 1500 i.e. and 3000 i.e. orally to deficient and rachitic rats respectively the fluxes were found to be 2.6 ± 0.3 (19) and 1.8 ± 0.3 (8) μMol/cm²h. Treatment of vitamin D deficient rats with 30 i.e. daily did not change the Ca-influx. The influx across the brush border membrane of Ca in the distal jejunum of rats kept on vitamin-D free diet is now influenced by vitamin-D administration.

- 89 Olsen I and E. Sjøgren (Department of Pharmacology and Toxicology Veterinary College of Norway Oslo Norway): INTESTINAL GLUCOSE TRANSPORT IN VITRO INFLUENCED BY CHLORHEXIDINE AND LAURILSULPHATE.

Recently it has been shown that considerable amounts of chlorhexidine and laurylsulphate from mouth rinses will be retained in the oral cavity and swallowed thereby exposing the gastrointestinal tract. The purpose of this study was to compare the effects of these drugs on an essential digestive process: glucose absorption.

Sacs of rat everted small intestine were filled with and suspended in glucose-containing Krebs-Ringer bicarbonate saline and incubated for 1 h at 37° C in the presence of chlorhexidine acetate or sodium laurylsulphate. Histological sections from the loops were made.

Except for 0.05 mM laurylsulphate which had a stimulating effect glucose transport was decreased progressively within the range of 0.01-1.00 mM chlorhexidine or laurylsulphate. On an equimolar basis chlorhexidine was the most potent inhibitor. A 50 % inhibition occurred at approximately 0.08 mM chlorhexidine and 0.40 mM laurylsulphate respectively. The extent of mucosal disintegration could be related to the degree of drug inhibitory effect.

It is concluded that chlorhexidine in oral therapeutics is unlikely to affect glucose absorption essentially more than the commonly used laurylsulphate since higher concentrations of the latter are employed.

- 90 Tindall, A.R. (Institute of Biology and Geology University of Tromsø Norway): Gastroliths in Norwegian grouse (*Lagopus lagopus*)

During the summer the stones in the gizzards of wild Norwegian grouse are smaller and more numerous than in winter. In late winter a few grouse have been found without stones in their gizzards. Captive grouse kept during the winter on standard grit are found to have stones typical of the summer-time throughout the year. This shows that the composition is determined by the availability of stones rather than by an internal selection mechanism. In a few grouse however which have died in captivity the gizzard was completely blocked with stones.

Experimental work has shown that when a domestic fowl is force-fed with glass beads some beads are excreted within the first few days others being retained for months. Gravity seems to play a part in the retention of stones in the gizzard.

Calcareous stones disappear within a few days presumably dissolved by the acidity of the stomach although stones shaken with a solution of HCl were not readily dissolved.

Contrary to isolated gastric mucosae from frogs those of mammals become hypoxic when gassed with oxygen at atmospheric pressure. A pressure of 3200 mm Hg is necessary for a complete oxygenation. A chamber has been constructed in which such a high oxygen tension can be maintained and still the usual procedures for the experiment can be carried out such as gas-bubbling, PD measurement, electrical shortcircuiting, continuous titration of the acid secretion with a pH-stat method and administration of drugs.

Isolated mucosae of rats have been used and found to function with a high secretory rate in this oxygen pressure system. The method is currently being used for a study of the histamine, pentagastrin, cyclic AMP and phosphodiesterase inhibitors on the gastric secretion.

In decerebrate and unanesthetized cats, spinalized at low thoracic level, the activity in primary muscle spindle afferents from the peroneus tertius or the extensor digitorum muscle was recorded in dorsal root filaments. Other muscles in the hind limb were denervated. The cutaneous component of the superficial peroneal nerve was left intact and so were the dorsal and ventral roots except for the dorsal root filament recorded from. In such preparations a reflex contraction of the muscle under study could be evoked by strongly pinching the paw. In most preparations the spindles were not unloaded by the contraction but instead their discharge was retained or even accelerated. This indicates a simultaneous outflow of skeletomotor and static fusimotor activity in the flexion reflex. Attempts were also made to test the dynamic sensitivity of the spindles by applying linear extension to the muscle before and during reflex contractions. It was consistently found that the dynamic burst of activity was not increased during the reflex. Essentially similar findings were obtained in preparations where extrafusal but not intrafusal contractions were eliminated by critical curarization. It is concluded that dynamic fusimotor activity is not significantly increased during the flexion reflex.

Dyhré-Poulsen E & Hounsgaard J (Laboratory of Behavioral & Physiology Institute of Neurophysiology University of Copenhagen Denmark): TRANSMISSION OF AFFERENT IMPULSES THROUGH THE LEMNISCAL SYSTEM DURING A CONDITIONED MOVEMENT IN THE MONKEY

Previously it has been shown that the amplitude of potentials in the medial lemniscus evoked by electrical stimuli applied to a cutaneous nerve in the cat is reduced during and before a movement of the limb. To determine whether a similar inhibition exists in primates as well monkeys were trained to depress a lever partially maintain a fixed posture and depress the lever fully at the onset of a tone. Electrical stimuli were applied to a peripheral nerve before and during the conditioned movement and potentials were recorded between a cross electrode in the medial lemniscus and an indifferent electrode.

The amplitude of the potential from the medial lemniscus was depressed during and before movements beginning around 200 ms before movement of the limb. As in the cat the decrease varies with the velocity of the limb. Furthermore the decrease varies inversely with the stimulus intensity. Stimuli just above threshold at rest evoke potentials that are more reduced during movement than potential evoked by stronger stimuli. This may be interpreted to indicate that the pool of inhibitory interneurons is constant or has a maximum and that the inhibition includes transmission through large possibly muscular afferents.

Johansson H & Silfvenius H (Dept of Physiology University of Umeå, 94 Sweden): DO THE DORSAL SPINOCEREBELLAR- AND THE HIND LIMB GROUP I CEREBRAL TRACTS HAVE COMMON SPINAL AXONS

It has recently been shown that spinal axons of the hind limb group I pathway projecting to the contralateral cerebral cortex ascend together with the axons of the dorsal spinocerebellar tract, DC (Landgren S & Silfvenius H J Physiol 1971; 218: 551). It is not known, however whether or not these two ascending tracts have common spinal axons. The problem is investigated in the present study using unitary recording from electrically activated bulbothalamic relay cells in the region of the medullary nucleus Z.

Three groups of neurones were observed: a) cells uninfluenced by electrical surface stimulation of the anterior cerebellar lobe ipsilateral to the spinal axons, b) cells activated at short latency from the anterior cerebellar lobe but not by collaterals of the DC, and c) cells activated at short latency from the anterior cerebellar lobe via collaterals of the DC.

It is concluded that the existence of relay cells in nucleus Z of type a and b favours the interpretation that the spinal component of the hind limb group I pathway to the contralateral cerebral cortex is not identical with that of the DC.

Bak, P. Klootwijk R. Miller S. and van der Burg J. (Department of 95
Anatomy, Erasmus University Rotterdam, Rotterdam, The Netherlands):
STEPPED PATTERNS OF NORMAL AND DEGENERATE CATS.

In normal cats trained to step on a treadmill all forms of stepping
can be described by two patterns: 1 ALTERNATE STEPPING, where hindlimbs
step alternately as in walking and trotting and 2 IN-PHASE STEPPING,
where hindlimbs step mainly together, as in galloping (Miller et al ,
J Physiol (Lond.) 1973 230 30P) In both patterns a constant time
coupling exists between the first extension of the hindlimb and flexion
of the ipsilateral forelimb. Similar stepping patterns with similar
coupling of limb movements can be induced by electrical stimulation of the
midbrain in degenerate cats stepping on a treadmill (technique of Shik
et al Biofizika 1966 11 659). The coupling of hind- and forelimb
movements is not abolished by bilateral section of dorsal roots between
L₂ and S₂.

From these results and from neurophysiological investigations of long
proprioceptive paths (Miller et al Brain Res. 1973 In Press) it is
suggested that the pattern of stepping and the co-ordination of the four
limbs are mainly organized in the spinal cord and are not primarily
dependent on hindlimb afferent input.

Jönasson, T. (Dept of Physiology Univ of Umeå, Sweden): CONVERGENCE FROM 96
THE MESENCEPHALON AND THE SPINAL CORD IN CLIMBING FIBRE PROJECTIONS TO THE
PARAMEDIAN LOBULE

Miller, Neelina and Oscarsson (Brain Res. 1969 14 234) showed that
electrical stimulation within two restricted areas of the mesencephalon
(Mes) evokes climbing fibre (CF) activity in the cerebellar anterior lobe
of the cat with the olivary neurones involved sharing also the hind limb
component of the dorsolateral spino-olivocerebellar path (DLF-SOCP).

The present investigation in halothane-anesthetized cat showed that
electrical stimulation in the region of the dorsal part of the red nucleus
(dorsal area of those mentioned above) evokes CF activity in three distinct
parts of the paramedian lobule (PM) namely caudally in the lateral (c-l)
and medial (c-m) thirds and rostrally in the medial (r-m) third. It was
demonstrated that the c-l projection shows convergence from Mes and the
hind limb DLF-SOCP. Furthermore when activating DF-SOCP (dorsal funiculus)
in C2 (both hind and fore limb components) a convergence from Mes to c-m
PM and r-m PM respectively could be shown. This would be expected because
the DF-SOCP by CF branching is known to project both to the anterior lobe
and to the c-m (hind limb) and r-m (fore limb) PM.

One explanation for this more extensive projection from Mes to the PM
is that in addition to branching CF there might be CFs destined solely
for the PM. This would result in a PM information partly different from
that to the anterior lobe.

Lukkari, J (Institute of Physiology University of Helsinki 97
Finland): DEVELOPMENT OF CEREBRO-CEREBELLAR AND CUTANEO-
CEREBELLAR RESPONSES IN GUINEA PIGS

The development of cerebellar responses to electrical stimulation of the parioral skin and cerebral cortex was studied in adult and new-born guinea pigs and intra-uterine fetuses. Cerebellar responses to ten electrical stimuli were recorded in the midline cortex of Larsell's lobules V-VI and averaged with a μ -Line computer.

In adults and new-borns the responses to peripheral stimuli consisted of five deflections the first of which was positive. In full-term fetuses only three and in 50-days fetuses only two such deflections could be seen. The latency of the first positive peak was 10 msec in adults and new-borns, 30 msec in full-term and 45 msec in 50-days fetuses.

The cerebellar responses to stimuli of primary sensory cortex were of the same shape as the responses to cutaneous stimulation. In adults and new-borns the latencies of the first positive peaks were 5-8 msec. In full-term fetuses they were 30 msec and in 50-days fetuses 60 msec.

From these studies it seems that the fast climbing fiber responses in guinea pig cerebellum develop at the time of birth, though the mossy fiber responses are present much earlier.

Secher, N, Ruberg Larsen, N, Binkhorst, R A and Bonde 98
Petersen, F (Laboratory for the Theory of Gymnastics
University of Copenhagen, Denmark) MAXIMUM AEROBIC POWER
DURING COMBINED ARM PLUS LEG EXERCISE RELATED TO THE STATE
OF ARM TRAINING

16 subjects (11 arm and two leg-trained athletes plus three non athletes) were measured for maximum oxygen uptake rate ($\dot{V}O_2$ max) and blood lactic acid during arm work, leg work and arm-plus leg work. $\dot{V}O_2$ max was in addition measured for some of the subjects during treadmill running and bicycling. Arm $\dot{V}O_2$ max was 55-119 % of leg $\dot{V}O_2$ max with an average of 85%. $\dot{V}O_2$ max during combined arm plus leg work was in average 6% higher than leg $\dot{V}O_2$ max (range 99-117 %). It is demonstrated that there exists a rectilinear relationship between the arm $\dot{V}O_2$ max and the combined $\dot{V}O_2$ max when both are expressed relative to leg $\dot{V}O_2$ max. Work output during combined arm plus leg work was in all subjects higher than during leg work. Aerobic and anaerobic metabolism contributed to this both being rectilinear related to the increase in work output. $\dot{V}O_2$ max measured during leg and arm plus leg exercise was similar to that measured during bicycling and treadmill running respectively.

Lammert, U (Inst for Physical Education Odense University 99
Denmark) THE EFFECT OF 30 MINUTES OF WORK WITH A CONSTANT
HEART RATE (70%) 0 1 2-3 4 5-6 or 7 TIMES A WEEK

The purpose of this investigation was to determine the relation between the effect of training and the training frequency when training intensity and duration were kept constant. The training frequency was either 0 1-2 3 4-5 6 or 7 times per week for 4 weeks. The training intensity was 70% of the aerobic power and the training duration was 30 min. After 4 weeks of training all subjects continued the training program 1 or 2 times per week for 8 weeks. 70% training intensity was defined as the workload on a bicycle ergometer producing a constant $HR = \{70\%(\max HR - \text{rest HR}) + \text{rest HR}\}$. Before and at the end of 4-8 and 12 weeks training a double control was performed on each of the subjects. The control consisted of 2 submaximal and 1 maximal workload on a bicycle ergometer. For each workload, HR, blood lactate concentration and oxygen uptake were measured.

The effect of training determined as the increase in training workload in maximal oxygen uptake in maximal ml O_2 /kg/min and the decreased HR and blood lactate concentration at 900 kpm/min was the same for a training frequency of 3 4 5 6 or 7 times per week. A training frequency of 2 times per week had less training effect than 3 times per week. The effect of 8 weeks of training at frequency of 1 or 2 times per week showed no systematic changes. The conclusion is that a training frequency of 3 times per week has a maximal effect at the intensity of 70%.

treize, S B and Aakvaag A (Laboratory of Physiology Norwegian College of Physical Education and Sport and Hormone and Isotope Laboratory Aker Hospital Oslo Norway): EFFECTS OF ANDROGENIC ANABOLIC STEROIDS ON STRENGTH DEVELOPMENT AND PLASMA TESTOSTERONE LEVELS IN NORMAL MALES

Androgenic-anabolic steroids have been used extensively by athletes to increase muscular strength and body weight. However, few controlled studies of the effect of such steroids on strength development have been done and the significance of the steroids in the training progress is still a controversial issue. Furthermore, no study has been reported regarding the consequences of steroid treatment on pituitary function in normal males.

To examine these effects, a physically homogeneous group of 22 healthy males, age 21 to 28, participated in a 8 week progressive weight training program during which half of the group received the active drug on a double-blind basis (Mesterolone) in doses of 75 and 150 mg/day respectively for the first and last 4 week period. All the subjects were given ca 30 g supplemental protein daily. The maximal strength (isometric) of four muscle groups was tested.

Although the steroid group showed gains over the controls in strength development, the difference between the groups was not significant. Highly significant suppression of plasma testosterone levels was observed in the steroid group, the suppression being most pronounced during the high dose administration.

If it is assumed that the plasma testosterone level reflects pituitary gonadotropin production, the results suggest that this androgenic anabolic steroid suppresses the pituitary.

- 101 Molbech S & S H Johansen (Danish National Association for Infantile Paralysis and the Department of Anesthesia II Gentofte Hospital 2900 Hellerup Denmark): ENDURANCE TIME IN SLOW AND FAST CONTRACTING MUSCLE GROUPS

The endurance time (the maximum duration of a sustained isometric muscle contraction) of a slow and a fast contracting muscle group was investigated in 5 subjects. The muscle groups examined were the plantar and the dorsal flexors of the ankle joint. The measurements were made with a 90° flexion in the knee joint. After determination of the maximum isometric muscle strength the endurance time was measured at 50 per cent of the maximum strength. The plantar flexors showed a statistically significant longer endurance time than did the dorsal flexors. This indicates that the ability of a muscle group to maintain a sustained isometric contraction depends on the relative proportion of slow and fast contracting muscle fibres.

- 102 Karlsson, J. (Dept of Physiology GIN Stockholm, Sweden): ISOMETRIC LOW EXERCISE AND GLYCOGEN DEPLETION IN FAST TWITCH (FT) AND SLOW TWITCH (ST) FIBRES IN HUMAN SKELETAL MUSCLE

Isometric tension in skeletal muscle is proportional to the number of motor units activated. Activation of motor units with different contractile and metabolic characteristics might explain the differences in endurance time and metabolic response at different levels of isometric tension. The present study was undertaken to determine the relationship between isometric tension and glycogen depletion patterns in human skeletal muscle fibres in an attempt to obtain some information about the type of fibres used at different tensions. Biopsy samples were taken from the vastus lateralis muscle of subjects at rest during and after varying programs of isometric work. Isometric tensions studied ranged from 10 to 40% of maximal voluntary contractile strength (MVC) of the knee extensors with the knee joint at 90°. Muscle glycogen was determined chemically and estimated histochemically with the PAS technique. At all tensions studied total glycogen declined but glycogen stores never were completely exhausted. At tensions below 20% of MVC a preferential glycogen depletion occurred in the ST (high-oxidative) fibres. When the tension exceeded 25% of MVC the FT (low-oxidative) fibres were the only fibres to become depleted of glycogen. It was concluded that at low isometric tensions in skeletal muscle only ST fibres were recruited. At higher tensions both fibre types were probably recruited with the FT fibres being the first to become depleted of their glycogen under these conditions.

A constant glycogen breakdown occurs in skeletal muscle during exercise. Whether this occurs more rapidly in one or the other fibre type may depend upon the relative work load or on the speed for doing the work. In the present study glycogen depletion patterns in human skeletal muscle fibres were followed during bicycle exercise with a pedal frequency of 60 rpm and loads requiring 30, 60 or 90% of $\dot{V}_{O_2 \text{ max}}$. Work was performed for 3 hr, 2 hr or to exhaustion at 30, 60 and 90% $\dot{V}_{O_2 \text{ max}}$, respectively. Total glycogen was determined chemically and estimated histochemically in biopsy samples from the vastus lateralis muscle taken at rest, during and after exercise. Glycogen degradation was 2.7 and 7.4 times faster when working at 60 and 90% $\dot{V}_{O_2 \text{ max}}$, respectively than at 30%. Slow twitch (high-oxidative) fibres were the first to lose glycogen (PAS stain) at all work intensities. As work continued there was a progressive glycogen depletion in the fast twitch (low-oxidative) fibres. Considerable quantities of glycogen remained in the muscle after 3 hr of work at 30% of $\dot{V}_{O_2 \text{ max}}$. This was found almost exclusively in the fast twitch fibres. Decreasing the pedal frequency to 30 rpm or increasing it to either 90 or 120 rpm while keeping total work output constant had little effect on the glycogen depletion pattern. These data demonstrate that the pattern of glycogen depletion in human skeletal muscle fibres during bicycle exercise is more dependent upon the relative (% of $\dot{V}_{O_2 \text{ max}}$) than absolute work load or the pedal speed for doing a constant amount of work. They also suggest a primary reliance upon slow twitch fibres during prolonged exercise.

Saltin B, B Essen and B Pernow (Dept of Physiology GIH Stockholm, 104 Sweden and Dept of Clinical Physiology Huddinge Sjukhus Huddinge Sweden): LOCAL FACTORS ENHANCING MUSCLE GLUCOSE UPTAKE DURING EXERCISE

The extent that blood-borne or local factors influence glucose uptake during exercise is unclear. This study was undertaken to analyze the effect of different muscle glycogen concentrations in the legs while work output and the supply of blood constituents were the same. Six men performed 2-leg exercise at 2 work loads demanding 2.6 and 3.5 l/min (63 and 83% of $\dot{V}_{O_2 \text{ max}}$) for 20 min each or to exhaustion twice with 1 hr of rest between. Due to one-leg bicycle exercise the day before and consuming a fat and protein diet different amounts of glycogen were available in the thigh muscle of the 2 legs at the start of the experiment (65 (a) vs 35 (b) mmol/kg). During 1 hr of rest between exercise sessions nicotinic acid was given to reduce the availability of FFA. After the first work bouts muscle glycogen was reduced to 18 (a) and 7 (b) mmol/kg. At the end of the experiment only 3-5 mmol/kg were left. From the start of exercise there was a net uptake of glucose in both legs averaging 0.03 (a) and 0.09 (b) mmol/l and during exercise a 8- and 5-fold increase was observed, respectively. This occurred inspite of a reduction in arterial glucose to 2.1 mmol/l. Glucose uptake was inversely related to muscle glycogen content and the percentage of glycogen filled fibres. Muscle glucose and G-6-P concentrations were lower in the leg with the lowest glycogen. It is concluded that the most rapid glucose uptake during exercise occurs in the muscle (fibres) with a low glycogen content. It is further suggested that this is brought about by a reduced local inhibition of hexokinase activity.

105 Lindholm, A. (Dept of Physiology OIH and Dept of Clinical Biochemistry Royal Veterinary College Stockholm, Sweden) METABOLIC RESPONSE AND MUSCLE METABOLITES DURING DIFFERENT EXERCISE INTENSITIES IN TROTTING HORSES

Horse racing is an important industry and popular recreational activity. The race horse is bred specifically for fast trotting and is a highly trained athlete. At present very little information is available about the metabolic response of these animals to exercise of different intensities and durations. The purpose of the present study was to describe changes in heart rate, muscle temperature and muscle concentrations of glycogen, triglycerides, lactate, ATP and CP in the trained trotting horse after varying regimens of exercise. Heart rate at rest was 36 beats \times min⁻¹ and it increased to 160 when the horse entered the track. During maximal trotting it reached 230-240 beats \times min⁻¹. The muscle temperature at rest was 36.9 °C which gradually increased to 41-42 °C after several bursts of trotting. Rectal temperature increased to as high as 40-41 °C. No marked change in muscle lactate occurred until trotting speed was within 10-15 seconds of maximal trotting speed (1.20 min \times km⁻¹). Values as high as 25 mM \times kg⁻¹ wet muscle were recorded. A marked muscle glycogen and triglyceride depletion was also observed. However the energy stores are never completely depleted. ATP and CP decreased in relation to the work load but the phosphagens never become depleted. The horse was found to be a good species for metabolic studies during exercise and it seems highly possible that the local increase in muscle lactate and/or muscle temperature limits the capacity for continuation of fast trotting.

106 H P Hlastala, B Wranne and C J Lenfant (Department of Medicine, University of Washington, Seattle, Washington 98195 and Natl Heart and Lung Inst of Health, Bethesda Md 20014, U.S.A.): PERIODIC CHANGES IN FUNCTIONAL RESIDUAL CAPACITY AND OTHER RESPIRATORY VARIABLES IN RESTING MAN

Periodic changes in oxygen uptake measured at the mouth do not necessarily reflect changes in alveolar oxygen exchange. They may also be due to changes in functional residual capacity (FRC), tidal volume (V_T) or respiratory period (T_R). This study was undertaken to determine the extent of such influences.

Breath-to-breath changes in FRC, $\dot{V}O_2$, $\dot{V}CO_2$, V_T , T_R and alveolar gas tension were monitored in resting recumbent male subjects. Changes in FRC were recorded using a body plethysmograph.

FRC showed an oscillating pattern with from two to seven predominant frequencies. The major oscillation had a period varying between 8.3 and 28 minutes from subject to subject with an amplitude varying from 42 to 176 ml. Other major oscillations were present at higher frequencies with lower amplitudes. Oscillations in $\dot{V}O_2$, $\dot{V}CO_2$, V_T , T_R , PAO_2 and $\dot{P}CO_2$ were less marked and with less distinct frequencies. $\dot{V}O_2$ and $\dot{P}CO_2$ were shown to be dependent on both FRC and V_T in terms of random changes but no strong relation was found in terms of periodic oscillations.

We conclude that a true steady state of FRC does not exist. This will influence breath-to-breath measurements of other respiratory variables such as $\dot{V}O_2$.

A warm-up program is performed by sportsmen empirically before a competition To elucidate the suitability of these procedures a study was made on 11 top-ranking runners and 14 master class skiers

A 10000 m race was run on two successive winter days with and without the warm-up period After the warm-up the mean result was better in seven cases (difference 18 to 160 sec) lower in one case and equal in three cases In 10 cases out of 11 the runners estimated that it was easier to run after a warm-up

Contradictory results were obtained in skiing experiments as a result of changing snow conditions In a 12 min skiing contest a better result was reached without a warm-up in 12 cases out of 14 The subjective estimation of the value of the warm-up was in agreement with the result only in four cases In a 15 km skiing contest no differences were observed when the time used for the first and second 5 km distance were compared as a function of the warm-up Subjective feelings were in favour of warming up

Warming up seems to include both a physical and a psychological component Too little is known as yet about its most effective methods and dosage

Peak rates of lactate formation in human skeletal muscle during intense dynamic or isometric exercise can be in the order of $0.4-0.5 \times 10^{-4}$ moles $\times g^{-1} \times min^{-1}$ The question has been raised as to how the glycolytic rate is regulated under these conditions The present study was undertaken to define the role for the conversion of phosphorylase (PLASE) α to PLASE β in human skeletal muscle during exercise Muscle biopsies from the vastus lateralis were taken at rest during and immediately after exercise and quickly frozen in liquid nitrogen PLASE was extracted from pulverized muscle samples in a glycerol- aqueous medium at temperatures below $-25^\circ C$ PLASE activity was analyzed fluorometrically from the production of $0-1-P$ in the presence (PLASE α) or absence (PLASE β) of 5-AMP (2 mM) Total PLASE ($\alpha + \beta$) activity ranged from $0.5-2.5 \times 10^{-3}$ moles $\times g^{-1} \times min^{-1}$ It was lowest in the muscles from endurance trained athletes and in muscles containing high concentrations of slow twitch fibres The PLASE activity was however at least 10-fold higher than peak rates of lactate formation At rest the ratio of PLASE $\alpha / (\alpha + \beta)$ was 17% Only small changes were observed following isometric or dynamic exercise In control experiments however electrical stimulation elevated the PLASE α activity to 25-30% in rat skeletal muscle It was concluded from these experiments that under rest conditions studied no major conversion of PLASE β to PLASE α took place These data suggest that the regulation of glycolysis in human skeletal muscle during exercise may be the result of some factor other than the conversion of PLASE β to PLASE α

- 109 Mäkelä-Hänen M, Hirvonen L and Peltonen T (Department of Physiology University of Oulu, Finland) LEG AND ARM EXERCISE TESTS IN CHILDREN

To study the development of physical fitness in children separate leg and arm exercise tests using a Werdnig ergometer (Ergomat W J Werdnig Lausanne) were performed in sitting position on 30 boys and 16 girls from an elementary school's fifth and sixth grades (mean ages 11 $\frac{1}{2}$ and 12 $\frac{1}{2}$ years respectively). A conventional bicycle ergometer test served as a control. In all tests the load was increased by 75 kpm/min at one minute intervals until exhaustion.

The maximum heart rate in the bicycle ergometer test was the same for both sexes and grades (188 per min). In the leg test with the Ergomat exhaustion was reached in a shorter time and with a smaller load than in the bicycle test. The maximum heart rate was about 25 beats per min lower in the Ergomat test. The increase in the systolic arterial pressure was equal in both types of leg test. In arm test the exhaustion stage was reached with a total work 10 to 15 per cent of that observed in the bicycle test. The maximum heart rate was lower than in the leg test with the Ergomat.

In the results for the fifth grade pupils no essential sex differences were observed. The sixth grade girls reached the exhaustion stage with a lower load than the boys.

- 110 Andén N E, Magnusson I and Stokk G (Department of Pharmacology University of Göteborg Sweden): BLOCKADE OF THE NERVE IMPULSES IN THE NIGRO-NEOSTRIATAL DOPAMINE NEURONS BY GAMMAHYDROXYBUTYRIC ACID AND GABA

The dopamine in the rat forebrain increased by about 70 per cent in 1 h both after axotomy and treatment with gammahydroxybutyric acid (sodium form 1.5 g/kg i.p.). Injections of 25% KCl but not 20% NaCl into the neostriatum prevented these increases in dopamine probably due to depolarization of the dopamine nerve terminals. Such a treatment with KCl also rapidly released the accumulated dopamine. Intrastriatal injections of tetrodotoxin caused an increase in brain dopamine similar to that seen after axotomy.

Injections of 25% KCl but not 20% NaCl into the substantia nigra induced an increase in brain dopamine to about the same degree as after axotomy. This effect may be due to a persistent depolarization of the dopamine cell bodies and a consequent blockade of the nerve impulse flow in the nigro-neostriatal dopamine neurons. Intraneural but not intrastriatal injections of GABA or gammahydroxybutyric acid also caused a marked increase in dopamine. The analogue beta-hydroxybutyric acid was ineffective. Gammahydroxybutyric acid may act by directly or indirectly mimicking an inhibitory GABA mechanism on the dopamine cell bodies in the substantia nigra.

Fuxe K Corrodi H Goldstein M BEkfelt T Lidbrink P and Ungerstedt U (Department of Histology Karolinska Institutet 104 01 Stockholm Sweden and Department of Psychiatry New York Medical Center New York U S A): NEW TYPES OF DOPAMINE RECEPTOR STIMULATING AGENTS

With the help of biochemical histochemical and functional studies on the nigro-neostriatal dopamine (DA) pathway in rats it has been possible to discover that 1-(2-pyrimidyl)-4-piperonylpiperazine (ET-495) and ergot alkaloids such as ergocornine and 28 α -ergocryptine (CB-154) are DA receptor stimulating agents with a prolonged action and could be new tools in the treatment of Parkinson's disease. These drugs induced a turning behaviour towards the intact side in rats with a unilateral 6-OH-DA induced degeneration of the nigro-neostriatal DA pathway. The effects were blocked by DA receptor blocking agents and reduced by a tyrosine hydroxylase inhibitor suggesting some dependency of presynaptic DA stores. Studies in rats with tremor following ventromedial tegmental lesions also show that ET-495 and CB-154 can cause relief of tremor activity. The studies on DA receptors suggested a dose-dependent decrease of turnover in the neostriatum and the limbic forebrain following ET-495, ergocornine and CB-154 treatment which supports the view that they are DA receptor stimulating agents.

Recent experiments with intraneostriatal injections of dibutyryl adenosine-3',5'-cyclic phosphate demonstrate a turning of the rat towards the intact side, an effect which was not blocked by DA receptor blockers. These results support the idea that activation of the DA receptor results in the formation of cyclic AMP which causes an activity change in the effector cell.

Avram A Antelman S and Ungerstedt U (Dept of Histology Karolinska Institutet Stockholm Sweden): ROTATIONAL BEHAVIOR AFTER CORTICAL LESIONS IN THE RAT

Amphetamine (1-5 mg/kg) induces rotational behavior towards the lesioned side in rats where the nigro-striatal dopamine (DA) system is unilaterally lesioned with 6-OH-DA. Apomorphine (0.25-2 mg/kg) induces rotation in the opposite direction. Amphetamine probably releases DA from the striatum while apomorphine stimulates supersensitive receptors in the denervated striatum (Ungerstedt 1971 Acta physiol scand 82: 1-10). In an attempt to study the functional interconnection between the striatum and the frontal cortex, unilateral lesions of the frontal cortex were made. It is known that lesions of the frontal cortex potentiate amphetamine excitation. In experiment 134 (214) Unilaterally lesioned rats were given amphetamine (2 mg/kg) (releasing DA) or apomorphine (2 mg/kg) (stimulating DA receptors) on the lesioned side. The results may be explained by an increased turnover of DA in the frontal lesion which is further potentiated by ergot alkaloids. The DA receptors thus making the opposite side more sensitive.

- 113 Christensen A V (Department of Pharmacology and Toxicology H Lundbeck & Co A/S Copenhagen Denmark): ACUTE AND DELAYED EFFECTS OF A SINGLE DOSE OF A NEUROLEPTIC DRUG

In mice neuroleptics are known to antagonize methylphenidate-induced stereotypy (Pedersen V and A V Christensen Acta Pharmacol et Toxicol 1972 31 484) and thymoleptics have been shown to intensify apomorphine-induced compulsive gnawing (Pedersen V Br J Pharmacol 1968 34 219P)

In the present study the effects of single doses of flupenthixol were followed over a period of 8 days. Every day after flupenthixol treatment groups of mice were tested according to the above mentioned methods. Complete or partial methylphenidate antagonism was recorded 1-2 days after flupenthixol administration. While not inducing compulsive gnawing in normal mice apomorphine did so 2-5 days after flupenthixol.

If methylphenidate antagonism is taken as an index of dopamine receptor blockade and the effect of apomorphine-induced behaviour as an index of receptor sensitization it may be concluded that a single dose of a neuroleptic drug causes a series of events in the neurone-receptor system initially characterized by receptor blockade and then followed by a receptor supersensitivity. Thus the effect of a single neuroleptic dose appears to be more complex and longer lasting than usually thought.

- 114 Kehr W, Carlsson A and Lindqvist M. (Department of Pharmacology University of Göteborg Sweden): EFFECTS OF CEREBRAL HEMISECTION ON FOREBRAIN CATECHOLAMINE SYNTHESIS

As previously reported interruption of the nerve impulse flow of the dopaminergic pathway results in a rapid and pronounced but short lasting increase of tyrosine hydroxylation followed by an increased accumulation of dopamine (Kehr W et al J Pharm Pharmacol 1972, 24 744). The effects of axotomy were further studied in different brain regions.

Axotomy was performed by means of a complete transverse cerebral hemisection in rats at the level of the caudal hypothalamus. Noradrenaline, dopamine, tyrosine and dopa were measured after injection of NSD 1015 (3-hydroxybenzyl hydroxylase HCl 100 mg/kg i.p.) an inhibitor of the aromatic amino acid decarboxylase. When measured directly after hemisection the accumulation of dopa on the lesioned side was most pronounced in the striatum while the levels in the cortex and limbic system were only slightly elevated if compared to the intact side. Two hours after hemisection the dopa levels on the lesioned side declined more rapidly in the limbic than in the striatal area however an increase was found in both regions on the intact side. After 1-3 days the tyrosine hydroxylation rate on the lesioned side declined even below control values accompanied by a drastic fall in dopamine concentration.

It is concluded that axotomy of dopaminergic neurones gives rise to an increase in tyrosine hydroxylation in the lesioned forebrain which may be due to a receptor-mediated feedback mechanism.

Ahl nius, S. Andén, N.-E. and Engel, J. (Department of Pharmacology Univ. of Gothenburg Sweden): RESTORATION OF LOCOMOTOR ACTIVITY IN MICE BY SMALL DOSES OF L-DOPA AFTER SUPPRESSION BY ALPHA-METHYLTYROSINE BUT NOT BY RESERPINE

The effects of low and high dose of L-DOPA on locomotor activity in mice were investigated after pretreatment with alpha-methyltyrosine or with reserpine. After pretreatment with alpha-methyltyrosine the administration of low doses of L-DOPA (10, 25 or 50 mg/kg i.p.) after inhibition of the peripheral L-DOPA decarboxylase restored the locomotor activity at 3 and 4 h concomitantly with a normalization of the brain catecholamine concentrations. On the other hand the same treatments with L-DOPA did not cause any reversal of the reserpine-induced suppression of locomotor activity despite a marked increase in brain dopamine. Both after pretreatment with alpha-methyltyrosine and with reserpine high doses of L-DOPA (200 or 400 mg/kg i.p.) induced a marked increase in locomotor activity even in comparison to saline-treated controls and other signs of overstimulation. The difference between the functional effect of the low doses of L-DOPA after pretreatment with alpha-methyltyrosine and with reserpine may be explained by a release of the newly formed catecholamine by nerve impulses from the nerve terminals only in the former case.

Aquilino, S.-M. Eckert, S.-L. and Sundvall, A. (Dept. of Neurology and Dept. of Pharmaceutical Pharmacology Univ. of Uppsala Sweden): TOPICAL LOCALIZATION OF CHOLINE ACETYLTRANSFERASE (ChAc) IN HUMAN EXTRAPYRAMIDAL NUCLEI

Indirect evidence suggests that the central cholinergic function is involved in parkinsonian symptoms and chorea (Duvoisin R. & Arch Neurol 1967 17:124; Aquilino S.-M. & Ström R. Life Sci 1971 10:403). As the extent of cholinergic innervation of a brain region should be reflected in its activity of ChAc and little knowledge of the ChAc distribution in human brain seemed of great importance. This report concerns the topical localization of the enzyme in brains divided 3-8 h post mortem from patients with pathology of neurological disease.

The caudate-thalamus-complex was dissected in six standardized frontal sections from the caudate head to nucleus (n) ruber and 3-5 mg specimens were punched out in about sixty localizations. Enzyme activity (nmol acetylcholine/g min) was determined by a modification of the radiochemical method described by Fonnum (Biochem J 1969 113:465).

The highest activities were found in putamen (186±8.7 n=28) and nucleus caudatus (131±4 n=42). A significant difference was found between lateral (17±2.0 n=9) and medial (6±1.0 n=7) globus pallidus. In the aforementioned structure the rostro-caudal distribution of ChAc was rather even. In the rostral section the nucleus caudatus however significantly lower activities were found than in the caudal sections in this nucleus. ChAc activities were slightly higher in thalamic nuclei (17.1±9 n=11) than in nucleus ruber (10±0 n=3), substantia nigra (14.1±7 n=11) and nucleus hypothalamicus (13±1.3 n=2). Studies are now extended to be influenced by extrapyramidal diseases.

117 Ljungberg T and Ungerstedt U (Department of Histology Karolinska Institutet Stockholm Sweden): 6-OH-DA INDUCED DA LESIONS: ADIPSIA APHAGIA SENSORY NEGLECT AND PHARMACOLOGICAL REVERSAL.

Bilateral lesions of the lateral hypothalamus induces a well-known syndrome of adipsia and aphagia (Ansd and Brobeck 1951) Ungerstedt (1971) found the same syndrome after 6-hydroxydopamine induced lesions of the nigro-striatal dopamine (DA) system and postulated that the lateral hypothalamic syndrome was due to lesions of the DA fibres to the striatum and not to lesions of an "eating-centre" in the lateral hypothalamus. We have now tried to substitute the lost DA by giving 6-OH DA denervated animals the DA receptor stimulating drug apomorphine. Bilateral DA lesions abolish as previously learned maze running for water reward. After a low dose of apomorphine the animal runs the maze and drinks the water. Furthermore a lesion of the DA pathway gives a sensory neglect to tactile olfactory visual and auditory stimuli on the side of the body contralateral to the lesion. This disability to orient towards sensory stimuli probably explains the adipsic and aphagic syndrome. The same sensory neglect has been found after lateral hypothalamic lesions (Marshall et al 1972). The reversal of adipsia and aphagia after apomorphine further supports the DA hypothesis.

118 Lorens, E. A. and Vale, H. (Institutes of Psychology and Physiology, University of Bergen, Norway): DISSOCIATION OF THE ANALGESIC AND REWARDING EFFECTS OF INTRACRANIAL STIMULATION. Bipolar electrodes were implanted in the lateral hypothalamus (n=10) or medial frontal cortex (n=10) of rats. The animals were trained to press a lever to deliver intracranially 1 sec trains of square waves. Self-stimulation current thresholds were obtained, and the animals tested at this current value 10 min /day for 10 consecutive days. The effect of stimulation on pain reactivity then was determined by measuring paw lick or jump latency following placement on a hot plate (55 C). The absence of either behavioral response for 45 sec indicated an analgesic effect of the stimulation. Stimulation of the lateral hypothalamic area produced both rewarding and analgesic effects. The current intensity necessary to produce analgesia, however, was significantly greater than that required to maintain self-stimulation behavior. Stimulation of the medial frontal cortex also yielded a positively reinforcing effect, but failed to induce analgesia regardless of the intensity or rate of stimulation administered. Stimulation of a brain site which yields a positively reinforcing effect thus will not necessarily induce analgesia. This suggests that different anatomical systems are involved in mediating the rewarding and analgesic effects of intracranial stimulation.

Knapp, T M Lubar J F , (Dept of Psychology Univ of Hous- 119
ton Houston Tx 77004; Dept Psych ,Univ of Tenn Knoxville
Tn p t Institute of Psychology Bergen): OPERANT CONDITIONING
OF 40 HZ ACTIVITY IN THE AMYGDALOID NUCLEI OF THE CAT

High frequency activity such as 40 Hz in the amygdala has
been associated with olfactory functions (Gault and Leaton Ele-
ctroenceph clin Neurophysiol 15 1963), emotional states (Leese
Psychiat Res Repts 12, 1980), and arousal (Delgado et al, Brain
Res 22 1970) Using cats with chronically implanted bipolar
electrodes located in the basolateral amygdala and medial fore-
brain bundle (MFB) each animal was reinforced for the occurren-
ce of 40 Hz amygdaloid activity Reinforcement consisted of
MFB stimulation (60 Hz, 0.5 sec 0.2-0.5 ma) beginning with a
CRF schedule and progressing to a FR 12 schedule After 56 days
of training 30 min per day the cats showed a sixfold increase
over initial 40 Hz levels These animals were also capable of
discriminating the differential contingencies under which they
were trained Following completion of this task the cats were
extinguished until their preconditioning baselines were recov-
ered Correlates of increased 40 Hz amygdala activity included
aggressiveness as manifested in difficulty in handling the ani-
mal by the experimenter altered emotionality and EEG time-
period changes

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Lubar

Livassy P J & Meyer P (Psychology Department University of Western 120
Australia): LEARNING A BRIGHTNESS DISCRIMINATION TASK UNDER CONDITIONS OF
ELECTRICAL STIMULATION OF THE DORSAL HIPPOCAMPUS IN THE RAT

Rats with electrodes in the CA1 area of the dorsal hippocampus given 0.5
sec train of electrical stimulation every 3.0 sec during training on a
brightness discrimination task failed to learn Following learning with-
out stimulation such stimulation led to a significant decline in correct
responses With 2.0 sec of stimulation 3.0 sec after each response
learning was gain disrupted but the stimulation did not affect performance
after learning was achieved This suggested interference with both
consolidation and retrieval processes (LIVASSY P & MEYER P Neuro-
psychologia 1973 11 75)

To further explore this possibility 3 groups each of 9 rats were trained
on the task One group was stimulated only during the period of exposure
to the stimuli the second group was given stimulation 10 sec after
response and the third group a control group with electrodes in the cortex
directly over the CA1 area was given 0.5 sec of stimulation every 3.0 sec
during training Group 1 animals showed disruption of learning and Group
2 animals showed some retardation in the rate of acquisition All animals
were brought to criterion and then were tested under 3 different
stimulation conditions 1 a "continuous stimulation stimulation during
stimulus presentation and stimulation 3 sec after response Disruptive
effects were observed in some but not all animals under conditions 1 and 2
The nature of these effects appeared to be dependent on the location of the
electrodes in relation to the CA1 area

121 Lidbrink, P (Dept of Histology Karolinska Institutet Stockholm Sweden):
ON THE ROLE OF CENTRAL NA NEURONS FOR REGULATION OF SLEEP AND WAKING

The sleep-waking cycle can be altered by limited lesions to either noradrenaline (NA) or 5-hydroxytryptamine (5-HT) neurons (Jourvet M Science 1969 163 32). Recent pharmacological studies indicate that the NA neurons are of importance for wakefulness (Jones B Brain Res 1972 39 121). To test the relationship between wakefulness and different NA neuron systems specific lesions of the NA pathways continuous recording of the EEG and subsequent evaluation of changes in NA levels have been performed.

Lesions of ascending NA axons from cell bodies in the brain stem were made by intracerebral injections of 6-hydroxydopamine. The EEG and neck EMG was continuously recorded for 4-8 days after the lesion. The recordings were scored for wakefulness and the different states of sleep. The effect of the lesion on the NA neurons was evaluated with fluorescence histochemistry and biochemical determinations of NA.

Lesions which caused disappearance of the NA nerve terminals in the cerebral cortex produced a decrease of wakefulness by about 25%. This time was replaced by a pattern of cortical deactivation with slow waves. The effect was more pronounced when the lesion also included the NA axons projecting to subcortical areas in the forebrain. Activation of the cortex during paradoxical sleep was not affected. The animals gradually recovered from the loss of wakefulness due to the lesion and after one month they showed a normal sleep-waking cycle.

The results suggest that NA neurons in the lower brain stem can directly influence the forebrain and are of importance for maintaining the activation of cortical activity during wakefulness.

122 Ägren G and Meyerson, B J (Dept of Med Pharmacology University of Uppsala Sweden): PREFERENCE FOR PAIR PARTNER IN THE MONGOLIAN GERBIL

Male and female gerbils were kept in pairs from about 90 days age and permitted at least one litter before the male was vasectomized. The preference of the female for her male pair-partner versus a strange male was tested. The apparatus consisted of three goal-cages each connected to a centrally placed cage by means of registering units which showed the location of the test-animal. One goal-cage held the pair-partner, another one strange male and the third goal cage was empty. The contact with the males was restricted by a wire-mesh. The time spent in the different goal-cages was recorded during a test-session of 3 hours. The tested subject was isolated for 3 hours before each test-session. One test-session was performed per day/animal for 5 consecutive days.

A preference for the pair-partner was seen for the pair-partner. Castration of the pair-partner reduced the preference whereas castration of the strange male did not significantly influence the preference. When the female was in diestrus the preference was higher than in estrus (cornified vaginal smears).

The preference of the male for his pair-partner was tested analogously and was related to the stage of estrous-cycle of both the pair and the strange female.

Ahlenius S and Engel J (Department of Pharmacy - University of Copenhagen, Denmark): A ROLE OF CENTRAL DOPAMINE IN AVOIDANCE BEHAVIOUR

The administration of a dopamine beta-hydroxylase inhibitor (bis (4-methyl-1 homopiperazinylthiocarbonyl)disulfide) induced a partial disruption of a conditioned avoidance response in mice. The CAR was suppressed to 75 per cent of control 24 h after the LA-63 treatment. The same dose of noradrenaline (NA) to about 35 per cent of control and to about 30 per cent of control. There was a slight increase in brain dopamine (DA) after administration.

The administration of DL-threo-3,4-dihydroxyamphetamine antagonized the LA-63-induced reduction. This partly counteracted the decrease in CAR and caused a decrease in brain DA.

From the present results it is suggested that the mediation of a CAR in mice

Buus Lassen, J (Department of Pharmacy - University of Copenhagen, Denmark): EFFECT OF P-CHLOROPHENYLENEAMINE ON MOTILITY IN RATS AFTER INHIBITION OF 5-HT STORAGE UPTAKE AND RECEPTOR INTERACTION

PCA has been shown to deplete brain 5-HT. Triocyclins, thymoleptics and chlorpheniramine antagonize this 5HT-depletion (Squires & Toxicol. 1972 31 suppl 1 35). In the present study the effect of PCA on motility of rats was studied using Animex motimeter. 2 rats were hyperactive consisting of abnormal head movements from side to side were also present.

The effect of PCA 5 mg/kg was investigated of substances interfering with 5-HT synthesis. The 5HT-synthesis inhibitor H 69/17 250 mg/kg, hydroxylase inhibitor H 44/68 250 mg/kg, 7.5 mg/kg imipramine, 3.1 mg/kg desipramine, 26 mg/kg amitriptyline, 1.7 mg/kg haloperidol 0.1 mg/kg antagonized PCA-hypermotility. H 44/68 dopamine (DA) β -hydroxylase inhibitor influence the effect of PCA.

These results indicate that 5HT hyperactivity and that 5HT-membrane transport of PCA into brain 5HT neurons

125 Brown, R. Engel J. Carlsson A (Department of Pharmacology University of Göteborg Sweden): EVIDENCE FOR CATECHOLAMINE INVOLVEMENT IN THE DISRUPTION OF BEHAVIOR DURING HYPOXIA

Hypoxia results in a decreased hydroxylation rate of tyrosine and tryptophan (Davis and Carlsson J Neurochem 20:913 1973). Two experiments were performed to determine if the disruption of catecholamine (CA) metabolism was of behavioral importance. In mice, the depression of locomotor activity under 12% oxygen was reversed by administration of apomorphine plus clonidine. These agents directly stimulate CA receptors. The locomotor activity under hypoxia was not increased by amphetamine, a drug which is believed to act by releasing newly synthesized CAs. However, if a low dose of L-DOPA plus a peripheral decarboxylase inhibitor was administered prior to amphetamine, the locomotor activity was stimulated. To examine the effect of hypoxia on a more specific behavior, rats were trained to avoid grid shock in a shuttle box. Avoidance responding was impaired by 8-65% oxygen, but the blockade was reversed by administration of L-DOPA plus a peripheral decarboxylase inhibitor. The data suggest that central CAs are involved in the behavioral disruption under hypoxia and that when transmitter precursor is supplied so that synthesis bypasses the oxygen-requiring rate-limiting step, central dopaminergic and possibly noradrenergic neurons are able to maintain behavior.

Persson, S-A and Wahlström, E (Department of Pharmacology University of Umeå Sweden): TUBE RUNNING ACTIVITY IN MICE TO EVALUATE THE EFFECTS OF DRUGS

The 5-Hydroxytryptophane (5-HTP) syndrome in mice includes hyperextended abducted hindlegs. The movements of the hindlegs are ineffective on a flat surface. This part of the syndrome has been used in a simple quantitative test.

One hour prior to the test, male mice (25-33 g) were put in a 120 cm plexi-glass tube with an inside diameter of 3.2 cm. At the time of testing, a gentle air stream was started blowing in the direction of the run. The mice were placed in the air inlet end of the tube and the time it took for the mice to run 100 cm was measured.

In preliminary experiments, mice pretreated with Nialamide (50 mg/kg i.p.) 2 h prior to 5-HTP (50 mg/kg i.p.) were tested every 15 minutes for 3 h after the last injection. They consistently passed the tube faster than Nialamide-saline treated controls (for instance 9.00 ± 1.51 (SE) s, N = 6 versus 129.5 ± 49.6 s, N = 6, $t = 2.43$, $P < 0.05$ two hours after the last injection). Pretreatment with lithium chloride (5 mg/kg i.p. 20 min prior to the 5-HTP) decreased the effect of 5-HTP (64.7 ± 18.4 s, N = 6, $t = 3.02$, $P = 0.02$).

In mice, the 5-HTP syndrome thus seems to facilitate the ability to pass through a tube.

Ranje Ch and Ungerstedt U (Dept of Psychology University 127 of Stockholm Sweden and Dept of Histology Karolinska Institutet Stockholm Sweden): EFFECTS OF CHRONIC AMPHETAMINE AND APOMORPHINE UNDER CONDITIONS OF HIGH MOTIVATION

Ten rats were fully trained to escape an underwater Y-maze by choosing the illuminated instead of the dark arm. Illumination was randomly changed between arms. The effect of chronic administration of d-amphetamine (6mg/kg 40 min before swimming) or apomorphine (5mg/kg 5 min before swimming) was investigated by testing for swimming time and errors after these drugs every second day and without drugs every other second day. Amphetamine treated animals fell into three groups on the basis of swimming time: animals unaffected by the drug, animals using 8 times longer and animals using 17 times longer swimming time than normals. There was an insignificant increase in number of errors. The apomorphine treated animals responded as a homogeneous group using 9 times longer swimming time than normals. They showed a significant increase of errors. The different effects of amphetamine and apomorphine may be due to their pharmacological effects: amphetamine releasing dopamine and norepinephrine, apomorphine stimulating dopamine receptors. The individual differences among the amphetamine animals are interesting in terms of the addictive properties of the drug.

Carrer H and Meyerson B J (Dept of Med Pharmacology University of 128 Uppsala Sweden): INFLUENCE OF LSD, APOMORPHINE AND MORGANINE SYNTHESIS INHIBITORS ON THE URGE TO OBTAIN WATER AND SEEK HETEROSEXUAL CONTACT IN THE FEMALE RAT

To study the influence of varying monoaminergic activity levels on motivation, a serotonin agonist (lysergic acid diethylamide, LSD), a dopamine agonist (apomorphine, APO) and monoamine synthesis inhibitor (p-chlorophenylalanine, PCPA and α -methyl-p-tyrosine, MT) was used. The effect of these agents on the urge to drink water in water deprived animals or to seek heterosexual contact in castrated oestrogen treated female rats was determined. Motivation was measured using the increasing barrier technique, i.e. the willingness to cross an electrical grid to reach water or a sexually stimulating. In parallel the influence on spontaneous motor activity (SMA) of the different treatments was recorded.

LSD 0.05 mg/kg but not 0.01 mg/kg significantly decreased the number of crossings when the goal cage held a male. The higher dose clearly depressed SMA. Apomorphine (0.05 or 0.02 mg/kg) decreased the number of crossings of water. Identical doses of APO significantly depressed SMA.

OMT 100 mg/kg decreased crossings to reach male but slightly increased crossings for water. PCPA was given for four consecutive days (100+50x3 mg/kg). PCPA treatment increased grid crossings by females reaching for the male; this effect was observed only in oestradiol benzoate treated subjects. The same treatment decreased crossings in water deprived animals seeking water.

¹Meyerson B J and Lindström L. Acta Physiol suppl 389 1973

- 129 Eliasson, M and Meyerson B J (Dept of Medical Pharmacology University of Uppsala Sweden): INFLUENCE OF LSD AND APOMORPHINE ON HORMONE-ACTIVATED COPULATORY BEHAVIOR IN THE FEMALE RAT

Increased monoaminergic activity decreases the estrogen+progesterone activated copulatory response (lordosis reflex) in female ovariectomized rats. An antagonistic action of serotonin has been proposed based upon effects of neuropharmacological agents with different actions on central nervous monoaminergic mechanisms.¹

The present investigation is concerned with the effects on the female copulatory response of LSD and apomorphine. Accumulating evidence suggests agonistic effects of LSD on central serotonin receptors and of apomorphine on dopamine receptors.

LSD 0.1 mg/kg significantly inhibited the lordosis reflex 10 min after injection. Higher doses had a more prolonged effect with complete recovery at 90 min. The effect of LSD was reduced by α -m-Tyrosine 200 mg/kg 5 hrs before LSD and prolonged by p-chloro-phenylalanine 350 mg/kg 5 hrs before LSD. Chlorpromazine 0.5 mg/kg 1 hr before did not influence the LSD effect but the dopamine antagonist pimozid 0.5 mg/kg 1 hr before reduced the inhibitory effect of LSD. Apomorphine 0.20 mg/kg significantly inhibited the lordosis reflex 10 min later with complete recovery at 60 min. Effect of a higher dose was not recovered until 120 min. This effect was prevented by treatment with pimozid 5 hrs before but was not influenced by α -m-Tyrosine pretreatment. The results will be discussed with regard to the implication of serotonergic and dopaminergic mechanisms in the inhibition of hormone activated copulatory responding in the female rat. Meyerson et al. In: Neuropharmacology, Proc VIII C N I P Congr Copenhagen 1972 in press.

- 130 Otto, U (ACO Läkemedel AB Box 3026 S-171 03 Solna 3 Sweden): THE ANALGESIC ACTIVITY AND ACUTE TOXICITY OF SODIUM SALICYLATE AND CODEINE PHOSPHATE COMBINATIONS IN RAT

The experimental animals were rats of Sprague-Dawley strain. Sodium salicylate and codeine phosphate and combinations of these drugs were administered subcutaneously.

The analgesic activity was determined by means of a technique described by Paulow (Acta Pharm Suecica 6 193 1969 & Ibid 6 207 1969) and later modified by Otto et al (To be published). In this method the tail of the rat is stimulated for 80 ms using a frequency of the electrical square waves of 125 Hz and a pulse-width of 1 ms. A nociceptive response is assumed to be produced when the animal squeaks. The pain threshold voltage for each animal is determined before administration of the drug. After injection of the drug the threshold is registered at 10 min intervals and the graded response is expressed as a percentage of the pre-treatment threshold voltage.

The LD₅₀ determinations were calculated at minimum three dose levels with at least three groups of rats. The mortality ratio was recorded 24 h after administration.

Results were obtained indicating that the analgesic effect of the combinations of sodium salicylate and codeine phosphate seemed to be additive. The toxicity of codeine phosphate however was found to be potentiated of sodium salicylate while the acute toxicity of sodium salicylate was not influenced by an addition of codeine phosphate.

Larsen J -J and Hermansen, K (Research Division of Pharmacia AS Hillerød Denmark): NEUROLEPTANALGESIA PRODUCED BY A COMBINATION OF ETORPHINE AND ACETYLPROMAZINE AND ITS REVERSAL BY AN ANTAGONIST DIPRENORPHINE IN THE DOG 131

The pharmacological actions of etorphine have been described previously by Blane G F et al (Br J Pharmacol 1967 30 11-22). Administration of etorphine and acetylpromazine in combination (Immobilon^R) produces a clinically useful neuroleptanalgesia in the dog which is readily antagonized by diprenorphine (Hansen H H and Szabo J Nord Vet -Med 1973 25 38-45).

The analgetic effect of Immobilon (etorphine 125 µg/ml and acetylpromazine 500 µg/ml) has been studied and compared with that of its two components and morphine. The following values were found (ED 50 µg/kg s.c.; acetic acid test mice): Immobilon (etorphine 0.3 + acetylpromazine 1.2); etorphine 1.2; acetylpromazine 40 and morphine 350.

The neuroleptanalgesia (0.05 ml/kg Immobilon i.v.) was evaluated by checking different reflexes in seven dogs. At the same time the cardiovascular action of Immobilon and its antagonist diprenorphine (Revivon^R) was followed by recording the systolic/diastolic blood pressure, the heart rate and the ECG. It is concluded that the intravenous administration of the combination of etorphine and acetylpromazine results in a clinically useful neuroleptanalgesia without side effects from the cardiovascular system prohibitive for its clinical use.

Wahlström, M (Department of Pharmacology University of Umeå Sweden): THE RELATION BETWEEN SEIZURE AND TOLERANCE IN THE ABSTINENCE SYNDROME AFTER CHRONIC BARBITAL TREATMENT IN THE RAT 132

Seizures as a symptom of physical dependence could be one way for the organism to decrease excitability in the CNS; normalize the functional state of the CNS. Such changes in excitability may be revealed by changes in tolerance.

Male rats were treated with barbitol in the drinking water (consumption: 190-260 mg/kg/day) for 4-5 months. The threshold dose of hexobarbital needed to obtain an EEG-criterion (Wahlström M. Acta pharmacol toxicol 1966 24 404) was used as a measure of tolerance 3 days after the end of the barbitol treatment. Seizures were induced by electrical stimulation 1 h prior to the threshold determination. The results (controls inside brackets) are given in per cent of a pre-experimental average.

Experimental treatment prior to test	Number of thresholds		Mean threshold per cent
	below 110 %	above 110 %	
Seizure	8 ^x (18)	10 (0)	119 (90)
No seizure	0 (17)	13 (1)	141 (89)

x) One rat had a spontaneous seizure 0.25 h prior to test.

A seizure could thus abolish tolerance in approximately 50 % of the barbitol treated animals. The hypothesis stated above has not been refuted by the present experiments.

- 133 Malmström, C O and Meyerson B J (Department of Medical Pharmacology Univ of Uppsala Sweden): EFFECTS OF AN ANTIESTROGEN MER-25 ON TESTOSTERONE-ACTIVATED COPULATORY BEHAVIOR IN THE CASTRATED MALE RAT

Male Wistar rats (purchased as Specific Pathogen Free) castrated as adults were maintained on a submaximal response level with respect to the percentage of subjects which displayed copulatory behavior with a receptive female (mountX) by means of testosterone propionate (TP 0.10 mg/kg/week s.c.) Copulatory tests were performed once weekly 2 days after the TP injection. The results obtained after an experimental treatment were compared with the pre-experimental response level.

An antiestrogen ethamoxy-triphatol (MER-25) 150 mg/kg given 2 hrs before the TP injection decreased mountX from 73% to 41% ($p < 0.01$, $N = 37$) discontinued TP treatment decreased mountX from 78% to 49% ($p < 0.001$, $N = 37$) while oil vehicle given 2 hrs before the TP injection did not affect the response. Furthermore it was found that estradiol benzoate 50 µg/kg could activate copulatory behavior in never-TP-treated non-mounting subjects.

The data are consistent with the hypothesis that testosterone is first converted to estrogen before copulatory activation is brought about.

- 134 Bums Lassen J (Department of Pharmacology A/S Ferrosan 2860 Søborg Denmark) EFFECT OF 4 α -DIETHYL-H-TYRAMINE (H 77/77) ON MOTILITY IN RATS AFTER INHIBITION OF MONOAMINE UPTAKE AND RECEPTOR INTERACTION

H 77/77 has been shown to deplete brain noradrenaline (NA) of rats and mice. Tricyclic thymoleptics were found to antagonize this NA-depletion (Carlsson et al. Europ J Pharmacol 1969; 3: 367-373). In the present investigation the effect of H 77/77 on motility of rats in a familiar cage was studied using Animox motimeter. 2.5 and 5 mg/kg s.c. induced hyperactivity consisting of locomotion, sniffing, rearing, different grooming movements and head twitch. These behavioral items are almost identical to those observed in untreated rats during exploratory activity in an unfamiliar cage.

The effect of H 77/77 5 mg/kg was investigated after s.c. treatment with substances interfering with central monoamine function. The tyrosine hydroxylase inhibitor H 44/68 (250 mg/kg), the dopamine- β -hydroxylase inhibitor FLA 63 (40 mg/kg), imipramine (0.7), desipramine (6.3), amitriptyline (0.1), chlorimipramine (1.5), protriptyline (0.7) and aceperone (1.1) antagonized H 77/77-hypermotility. The serotonin synthesis inhibitor H 69/17 (200) did not influence the effect of H 77/77.

These results indicate that release of brain NA is involved in the H 77/77 induced hyperactivity and that NA-membrane blocking thymoleptics inhibit the uptake of H 77/77 into brain NA neurons.

To investigate the effect of changes in distal tubular sodium delivery on glomerular filtration the following experiments were performed on rats. Glomerular capillary pressure was estimated from proximal tubular stop flow pressure (SFP) measured with a servo null recording system proximal to an injected highly viscous silicone oil block. Distal to the oil block the late proximal segment was perfused either with Ringer or 300 mM mannitol solution at flow rates above and below the normal of about 13 nl/min. When the distal nephron was perfused with Ringer zero flow SFP was $53.3 \text{ cm H}_2\text{O} \pm 5.2$ (SD) and the decrease in SFP at 13, 27 and 43 nl/min were 0.1 ± 0.3 , 7.0 ± 4.4 ($p < 0.001$), $10.5 \pm 4.0 \text{ cm H}_2\text{O}$ respectively. When mannitol was the perfusing fluid there were no significant differences in SFP at different flow rates. It is suggested that increase in distal sodium delivery gives rise to an increase in afferent arteriolar resistance whereupon the glomerular filtration rate decreases. At reduced distal delivery the intrarenal regulation is either abolished or may involve changes in the resistance of both afferent and efferent arterioles.

Ågerup B (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) THE EFFECT OF CHANGES IN THE PERITUBULAR PHYSICAL FORCES ON NET FLUID TRANSFER IN THE RATS KIDNEY PROXIMAL TUBULE

Changes in transubular net fluid transport have previously been induced by altering the intratubular hydrostatic and oncotic forces. These experiments seem to indicate a significant role for the existing physical forces on normal tubular reabsorption (A H G Persson et al. *Kidney Int.* 1972, 2, 203). To test whether these findings were valid when alterations were made at the peritubular side of the nephron the following set of experiments were undertaken.

Reabsorptive rate was measured from sequences photographs of a filtrate drop let enclosed between two oilcolumns in a straight surface proximal tubule. The tubular surrounding was perfused in two different ways. A Perfusion of the capillaries in the star vessel at 250 ml/min with ultrafiltrate plus 10 g% albumin. B Perfusion of the subcapsular interstitium ultrafiltrate plus 10 g% albumin.

The difference in transubular fluid flow between control and perfusion expressed as $\text{nl/mm}^2 \text{ sec}$ was:

A: $+0.069 \pm 0.065$ $p < 0.001$ controls 0.405 ± 0.090 $n = 16$

B: $+0.012 \pm 0.101$ $p > 0.5$ controls 0.458 ± 0.098 $n = 20$

These experiments showed that an increased capillary oncotic pressure induced a significantly increased tubular reabsorption. However an increased subcapsular interstitial oncotic pressure did not effect tubular reabsorption. A direct tubulo-capillary transport route is consequently suggested.

- 137 Røder M, Osvik P, Jr. and Kill F. (Institute for Experimental Medical Research Ullevål Hospital Oslo Norway) MECHANISM OF MAINTAINED GLOMERULAR FILTRATION RATE AT HIGH URETERAL PRESSURE.

Glomerular filtration rate (GFR) is maintained when ureteral pressure is raised on account of afferent arteriolar dilatation. On the other hand, GFR remains constant during a rise in renal perfusion pressure (RPP) on account of afferent arteriolar constriction. To examine if the relationship between GFR and ureteral pressure is influenced by RPP the effect of ureteral obstruction on GFR was studied at two RPP in 6 anesthetized dogs. RPP was elevated by carotid constriction. GFR was estimated as $^{51}\text{Cr-EDTA}$ extraction.

At control RPP averaging 127 ± 5 mm Hg GFR exceeded $91 \pm 8\%$ of control up to ureteral pressure of 45 ± 1 mm Hg. At high RPP averaging 183 ± 4 mm Hg GFR exceeded $96 \pm 2\%$ up to ureteral pressure of 80 ± 2 mm Hg. At ureteral pressures exceeding 50 mm Hg GFR was significantly lower ($p < 0.05$) at control RPP than at high RPP.

Thus the vasoconstrictive effect on afferent arterioles induced by raising RPP can be partly compensated by raising ureteral pressure suggesting autoregulation of the transmural pressure of the afferent arterioles.

- 138 Eide I, Løyning E and Kill F. (Institute for Experimental Medical Research University of Oslo Ullevål Hospital Norway) THE EFFECT OF ATHERIC β -RECEPTOR STIMULATION ON RENIN RELEASE DURING REDUCED RENAL PERFUSION PRESSURE.

The mechanical stimulus for renin release during constriction of the renal artery appears to be autoregulated dilatation of afferent arterioles (Eide I, Løyning E. and Kill F. *Circulat Res* 1973 32: 237). Renin release may also be raised by stimulation of β -receptors, but the interrelationship between the two types of stimuli remains unclear.

In ten dogs intrarenal infusion of isoproterenol at $4.0 \mu\text{g}$ per min increased the secretion of renin from 1.8 ± 1.5 (mean \pm SD) to $8.9 \pm 5.3 \mu\text{g}$ per min ($p < 0.001$). Similarly, during standardized reductions of perfusion pressure below the range of autoregulation of renal blood flow isoproterenol increased renin release by 70% from 31.9 ± 10.2 to $54.3 \pm 15.7 \mu\text{g}$ per min ($p < 0.001$). This increase is larger than at control perfusion pressure ($p < 0.01$). Conversely β -receptor blockade with propranolol in five dogs reduced renin release by 38% from 47.6 ± 28.0 (range 25.1 - 93.7) to $29.5 \pm 10.5 \mu\text{g}$ per min (reduced in four and unaltered in one dog).

Thus stimulation of β receptors increases renin release/at low than at control perfusion pressure. β -receptor activity became therefore an effective regulator of renin release at reduced renal perfusion pressure.

The regulation of proximal tubular reabsorption of sodium is essential in maintaining water and electrolyte balance in the body. Several attempts have been made to find substances that decrease sodium reabsorption in the proximal tubules. Among the substances tested is bradykinin which has been shown to increase the urinary output of sodium. This study was undertaken to find out whether the increased output is due to an effect on the proximal tubular reabsorption of bradykinin.

On Inactin[®]-anaesthetized rats the left kidney was prepared for micro-puncture. A split-oil droplet technique was used to determine tubular reabsorption by time-sequenced microphotography. From the photographs the $t_{1/2}$ for the droplets was determined and used as a measure of tubular reabsorption. Thereafter a cannula was inserted into an adjacent star vessel and perfusion of the peritubular capillary network was started. During this perfusion another determination of the tubular reabsorption was done. In one series the perfusate was a modified Ringers solution. In another series 0.9 micrograms per ml of bradykinin was added to the perfusate. The perfusion rate varied between 400 and 900 nl/min. Results: first series control $t_{1/2}$ 18.3 sec perf $t_{1/2}$ 18.6 sec. Second series control 18.6 sec perf 26.9 sec. This increase of about 50% in $t_{1/2}$ during bradykinin perfusion is interpreted as a direct influence of bradykinin on the proximal tubular reabsorption as compared to Ringer perfusions where no change was seen.

THE USE OF ¹⁴C- AND ³H-LABELLED DERIVATIVES OF INULIN AS TRACERS OF INULIN IN VIVO

Radioactive derivatives of inulin (¹⁴CH₂OH- ¹⁴COOH- or C¹⁴H₃O-inulin) were infused together with native inulin and creatinine into anaesthetized rabbits. It was found that the renal clearance values of the labelled derivatives did not differ from those of unlabelled inulin and creatinine. Furthermore the specific activity of plasma and urine was the same as that of the infusion fluid. The plasma decay curves of radioactive and native inulin after withdrawal of the substances from the infusion fluid coincided and similar values were calculated for the distribution volumes of these substances (appr 26 %). By contrast the decline of plasma creatinine was slower than that of inulin and the distribution volume was found to be 45 %. Gel filtration analysis indicated the presence of varying degrees of low molecular weight contaminants in the labelled derivatives of inulin. It is concluded that in spite of the presence of impurities radioactive derivatives may be used as a substitute of native inulin for determination of glomerular filtration rate and extracellular space in the rabbit.

- 141 Ulfendahl H R Pinter G G Atkins J L Wolgast M and Ågerup B
(Inst of Physiol and Med Biophys Univ of Uppsala and Dept of
Physiol Univ of Maryland Baltimore Md USA):
TOTAL LYMPH FLOW OF RAT KIDNEY

To determine whether the flow of lymph plays a significant role in the fluid balance of the kidney and to estimate the rate of lymphatic drainage of extravascular plasma proteins from renal tissue we have attempted to measure total renal lymph flow (TRLF) in rats. We observed that a partial occlusion of the left renal vein (PORV) brought about an immediate and large increase in the flow of a main abdominal lymph duct (ALD) cannulated next to the aortic hiatus of the diaphragm. Moreover we found that occasionally in Sprague Dawley rats small lymphatics of the renal hilum appeared to join together into a single major renal lymphatic vessel. When lymph flow from such a vessel was diverted into a separate collecting cannula flow from ALD decreased and failed to respond to PORV. Based on these observations in preliminary experiments we estimated TRLF either as the difference in ALD flow before and after complete occlusion of the renal hilum (O Morchoe and O Morchoe J. Physiol. /London/ 1968 194 305) or calculated it by using the responses to PORV that occurred simultaneously in flows of a cannulated renal hilar lymph vessel and ALD. In rats weighing 200-350 gms that had received no fluid load we obtained a value of 2 to 3 $\mu\text{l}/\text{min}/\text{gm}$ of kidney which we consider to be an approximate estimate rather than an accurate measure of the total renal lymph drainage.

- 142 Tindall, A R (Institute of Biology and Geology University of Tromsø Norway) The activity of the rat ureter

The electrical and mechanical activities of the ureter have been recorded in situ by external recording devices. Usually the electrical and mechanical activities are closely correlated progressing from the renal end of the ureter. The frequency of contractions is about 25/min and the rate of propagation of the electrical activity is about 20mm/sec.

The normal electromyogram probably arises in the first 5 mm of the renal end of the ureter and retrograde activity may arise from a similar region at the vesicular end of the ureter. A close arterial injection technique shows that the proximal end of the ureter behaves as if it possess α receptors while the distal end behaves as if it had beta receptors.

Wallin, G., Dalius, W. *Experiments in the
ments of Clinical Neurophysiology, Scand
dic Hospital Uppsala Sweden*
PATHETIC OUTFLOW TO SKELETAL MUSCLES

The microneurographic study of the
muscle nerve sympathetic activity in
types of heart arrhythmias. Simultaneous
arterial blood pressure showed a
lay of 1 2 1 4 seconds was consistent
note correlation between the
occurrence of multiunit sympathetic activity
corresponded to diastoles and occurred
during pulse intervals of long
pressures. The relationship between
interval (or the end diastolic pressure)
occurrence of the bursts was a
shaped curve. An approximately
between the duration of the bursts
of the bursts. The results confirm
ting a strong baroreflex controlling
the vascular bed of skeletal muscles
plain previous findings of regulation of
vascular resistance in cases of

Blix A S H Refsum E L Gautvik

(Institute of Aviation Medicine
Blindern Oslo Institute of
Zoophysiology University of
Hospital Oslo Norway)

VASOCONSTRICTION IN THE DIVER

The effects of alpha-adrenergic and
parasympathetic (atropin) blockade
response to diving in the dog.

With no significant reduction
in heart rate alpha adrenergic
vasodilatation and virtually
of profound bradycardia during

By both in vitro and in vivo
ified that these effects could
interference of phenoxybenzamine
control.

Atropinization eliminated
cardia while the arterial pressure
creased suggesting that the
striator fibres was maintained.

It is concluded that the
tion of the initial diving
secondary reflex adjustment

- 143 Henriksen O & W P Paaske (Department of Nuclear Medicine Rigshospitalet Copenhagen Denmark & Institute of Medical Physiology M University of Copenhagen Denmark): INFLUENCE OF ORTHOSTATIC BLOOD PRESSURE CHANGES ON BLOOD FLOW IN SUBCUTANEOUS ADIPOSE TISSUE OF THE CRUS

The nature of autoregulation of blood flow in subcutaneous adipose tissue of the crus was investigated by means of the local xenon-133 washout technique. Orthostatic blood pressure changes were obtained by passive displacement of the leg in relation to the heart.

Blood flow remained constant from 20 cm below to 20 cm above the heart. Outside this range blood flow decreased. A decrement of blood flow with the leg in dependent position indicates an increase of vascular resistance as arterial and venous pressures increase in parallel below the heart.

Patients suffering from intermittent claudication get notable relief of pain when they place their legs in dependent position. This apparent increase of blood flow during dependency in these patients cannot be ascribed to an increase of the perfusion pressure head but rather by assuming that the vasculature in ischemic areas is paralyzed which might implicate a redistribution of blood flow from healthy areas with intact autoregulatory ability to ischemic regions creating an inverse Steal Syndrome.

- 146 Eklund B and L Kaijser (Department of Clinical Physiology Karolinska Hospital Stockholm Sweden): CHANGES IN REACTIVITY OF THE MUSCLE VASCULARITY WITH THE DURATION OF EXERCISE

The increase in muscle blood flow during work is considered due to local chemical environment. During prolonged work however blood flow is maintained in spite of a return towards resting levels of factors such as PCO_2 , osmolality, H^+ , K^+ and lactate in the effluent venous blood. To investigate if prolonged work changes the reactivity to vasoactive stimulation the effect on forearm blood flow of a infusion of adrenergic vasoactive substances such as noradrenaline, isoprenaline and phenolamine and of sympathetic stimulation (lower body low pressure LBLP) was studied after 5 and 55 min dynamic forearm work. Blood flow was measured with venous occlusion plethysmography.

No adrenaline decreased flow both after short and prolonged work but in connection with prolonged work a pronounced increase was seen on termination of infusion which could be reduced by β adrenergic blockade. It was not seen after LBLP. Isoprenaline increased flow more after short than prolonged work. Phenolamine increased flow markedly after prolonged but not after short work. During work no adrenaline decreased SO_2 of the effluent venous blood more during prolonged than short work indicating a more pronounced flow reduction.

It is concluded that prolonged work changes the vascular reactivity to such an extent that it might be of importance for flow regulation.

To elucidate whether vibrations in the cardiovascular system might exert a direct inhibitory action on smooth muscle contraction in blood vessels (a g post stenotic dilatation) we have characterized the influence of longitudinal sine-wave oscillations on contractile activity of isolated preparations of the rat portal vein and spiral strips of the rabbit thoracic aorta. Low amplitude vibrations over a wide range of frequencies caused an almost instantaneous decrease in active force. No decrease in passive tension was obtained when inactive tissues were vibrated. The relationship between vibration rate, amplitude and the relative inhibition was determined. At a frequency of 200 Hz a 25 per cent decrease in active force of portal vein spontaneous activity and high K^+ contracture and of aortic noradrenaline and K^+ responses were obtained with vibration amplitudes of 2, 1, 8 and 6 per cent of tissue length respectively. The results thus show that the oscillations cause prompt and pronounced inhibitions, most likely due to a direct action on the contractile elements. It seems probable that turbulence induced vibrations can influence vascular tone in some pathological conditions (arterial stenosis, A-V and stenosis) and it is suggested that an inhibitory response to pulse wave oscillations might have a physiological significance in regulation of conduit blood vessel diameter. (Supported by SMF 14X-3884)

Pyykkö I and J. Hyvärinen (Institute of Physiology
University of Helsinki, Finland) STUDIES ON FINNISH LUMBER-
JACKS ABOUT THE PHYSIOLOGICAL MECHANISM THAT LEADS TO
TRAUMATIC VASOSPASTIC DISEASE (TVD)

148

TVD is wide spread among Finnish chain saw operating lumberjacks. In modern chain saws the dominant frequency of vibration is around 125 Hz. In chain saws equipped with vibration dampers the peak-to-peak vibration amplitude of the rear handle is only 50-300 μ . In this frequency and amplitude region the pacinian corpuscles are exquisitely sensitive to vibration. Vibration of a limb also causes discharge in abdominal sympathetic ganglia of the cat (Miyamoto, J and J. Alanis, Jap. J. Physiol., 1970, 20: 725). On the other hand, vibration of muscles leads to the tonic vibration reflex (TVR).

We recorded finger pulse volume, TVR, and galvanic skin response (GSR) in 43 chain saw operating lumberjacks with TVD. Vasospastic reactions were provoked by body cooling and chain saw noise and particularly by vibration of hand. At different vibration frequencies the threshold amplitude that produced vasospasms runs parallel and 20 dB above the threshold of pacinian corpuscles. TVR and GSR appeared less directly related to the vasospasms. Clonidine appeared to have a beneficial effect on the vasospasms in some patients. We suggest that TVD is produced through prolonged excessive activation of a sympathetic vasoconstrictor reflex triggered by activity in pacinian afferents.

- 153 Ek L, Dahlström E, Carlsson E, Lissander B, Martner J and Åblad, B. (Dept of Pharmacology AB Hässle Mölndal and Dept of Physiology University of Gothenburg Sweden): HEMODYNAMIC EFFECTS OF TWO ADRENERGIC BETA RECEPTOR ANTAGONISTS IN CONSCIOUS DOGS. In the intact organism the acute cardiovascular effects of beta receptor blockade are probably mainly due to inhibition of adrenergic control of cardiac rate and contractility and to inhibition of the vasodilator effect of circulating adrenaline. / Lately a new cardioselective beta receptor antagonist H 93/26 has been reported (Åblad et al Life Sci 12:107-119 1973). As regards blockade of the cardiac response to cardiac sympathetic nerve stimulation H 93/26 was equipotent to propranolol while propranolol was a considerably more potent inhibitor of vasodilator effects of injected adrenaline than H 93/26. In anaesthetized cats and dogs the pressor response to adrenaline was potentiated by propranolol only. / Because of these findings the two compounds may be anticipated to produce differentiated hemodynamic effects in physiological conditions where endogenous adrenaline is of importance for the cardiovascular control. To study this question the acute cardiovascular effect of propranolol and H 93/26 have been compared in conscious dogs with chronically implanted devices under various conditions: at rest during exercise and in defence alarm situations. It was found that during defence alarm situations the hemodynamic actions of the two beta receptor antagonist differed indicating a release of adrenaline.

- 154 Nilsson S and D Grove (Department of Zoophysiology University of Göteborg Sweden and Marine Laboratories Penarth Bridge Wales): AUTONOMIC NERVE CONTROL OF THE SPLEEN IN A FISH GADUS MORHUA. Measurements of flow through the perfused spleen at constant pressure show that splanchnic nerve stimulation acetylcholine noradrenaline adrenaline and phenylephrine but not isoprenaline increase resistance. Quantitative studies show that cholinergic responses are selectively blocked by atropine and adrenergic responses by yohimbine or phentolamine. Splanchnic nerve effects are rarely completely blocked by adrenergic or cholinergic antagonists alone. Simultaneous adrenergic and cholinergic blockade regularly abolishes nerve responses. Where reduced responses to splanchnic nerve stimulation were obtained following 6-hydroxydopamine or reserpine treatment these could be abolished by atropine alone. Ganglion blocking agent (mecamylamine or hexamethonium) did not affect the responses to nerve stimulation. It is concluded that the spleen of Gadus is innervated by both cholinergic and adrenergic postganglionic constrictor fibres running in the splanchnic nerve.

FAGRELL H (Departments of Medicine and Clinical Physiology Karolinska 15.
Institutet Seraflimerlasarettet Stockholm Sweden) VITAL CAPILLARY MIC-
ROSCOPY - A METHOD FOR EVALUATING THE EFFECT OF THERAPEUTIC
PROCEDURES ON ISCHEMIC SKIN ULCERS

It has been shown that structural changes of the skin capillaries - as stu-
died by vital capillaroscopy (VC) - yield valuable information regarding the
viability of the skin in patients with arterial vascular disorders thereby
providing a means of estimating the potential risk for skin necrosis. It has
also been found that VC is of value for estimating the effect of therapeutic
procedures on the nutritional skin circulation in patients with ischemic skin
ulcers (Fagrell H Scand J clin Lab Invest Suppl 133, 1973)

In 1966 a new substance pyridinoloarbutamate (PC) (a bradykinin antagon-
ist) was reported to prevent vascular injury induced by atherogenic sub-
stances e.g. cholesterol (Shimamoto T et al Amer Heart J 71 216 1970).
The present study was undertaken to evaluate the effect of PC on the struc-
tural appearance of the nutritional capillaries of the skin surrounding ische-
mic skin ulcers and to assess the possibility of using VC for predicting
the prognosis of such ulcers.

It was found that in 5 out of 11 patients with skin lesions where no or
only a few capillaries were seen at the rim of the ulcers at the start of the
treatment an almost normal number of blood-filled capillaries appeared
after 11 weeks of treatment. A marked improvement of the skin lesions was
observed in these patients. In the only patient where the capillary bed did
not improve during the treatment the ulcer progressed successively.

PAAKKE W F O Henriksen P Sejrsen & H Levin Nielsen 156
(Institute of Medical Physiology B University of Copenhagen
Denmark): AUTOREGULATION OF BLOOD FLOW IN HUMAN CUTANEOUS AND
ADIPOSE TISSUES

The presence of autoregulation of blood flow was investiga-
ted in subcutaneous adipose tissue of the distal forearm and
in cutaneous tissue of the skin fold between thumb and fore-
finger by means of local xanox-133 washout techniques. Ortho-
static blood pressure changes were obtained by passive dis-
placement of the area under study to various test levels above
and below the reference level the jugular notch.

Blood flow in both tissues remained constant for all sub-
jects in an interval about the heart level. By elevation of
the arm outside the autoregulatory range blood flow was found
to decrease in proportion to the reduction in perfusion pres-
sure. By extreme downwards dislocation blood flow was suddenly
reduced to about 60 per cent of the reference value. Further
lowering did not reduce blood flow any further.

It is concluded that autoregulation of blood flow is present
in cutaneous and adipose tissue in the interval from 5 cm be-
low to 20 cm above the reference level as venous pressure is
constant and arterial pressure is decreasing 20 mm Hg in this
range.

157 Bø E. A. Hauge and B. A. Waaler (Institute of Physiology University of Oslo, Norway) THE SITE OF MECHANICALLY INDUCED CHANGES IN PULMONARY BLOOD VOLUME AND EFFECTS ON LUNG COMPLIANCE

Acute respiratory failure is frequently characterized by low lung airway compliance (C_L) increase in pulmonary blood volume (PBV) and interstitial edema. Causal relationships are claimed to exist between these lung parameters. Detailed mechanisms of interaction remain however to be elucidated.

We have investigated the relative effects of lung edema and increases in PBV on C_L and also the effects of separate rises in arterial and venous pressures on PBV and C_L .

In an isolated perfused and ventilated rabbit lung preparation lung weight and dynamic lung compliance were continuously recorded. A rapid 50% rise in PBV induced by elevation of left atrial pressure (P_{LA}) at constant flow caused a simultaneous 30% fall in C_L . With maintained high vascular pressure interstitial edema accumulated with no further fall in C_L . When inflow pressure was elevated to the same levels 1) by a rise in flow at constant P_{LA} and 2) by a rise in P_{LA} at constant flow the latter type of experiment gave an increase in PBV 3 to 5 times larger than the first one. The fall in C_L was closely related to the rise in PBV regardless of how such a rise was obtained. The major part of the lung vascular capacitance changes took place in venous vessels.

58 Åhrén, K., Jansson, P. O. and Selstam, U. (Department of Physiology and Department of Obstetrics and Gynecology University of Göteborg, Sweden): PASSAGE OF MICROSPHERES IN FOLLICULAR AND LUTEAL RABBIT OVARIES.

Little is known about vascular mechanisms in the ovary during different endocrine conditions. Regarding the ovarian blood flow it has been reported in the literature that direct measurement methods yield about ten times higher values than indirect methods.

In order to test whether these differences could be due to arterio-venous anastomoses the passage of $15 \pm 5 \mu$ non-radioactive microspheres was investigated using *in situ* perfusion techniques. In the present study virgin Swedish Land rabbits, 5-6 months old were used. Ovulation was induced by intravenous injection of human chorionic gonadotrophin.

In one group of animals the passage of microspheres across the ovary was quantified by perfusing both ovaries *in situ* at 75-mm Hg with a 6% dextran solution holding a known concentration of microspheres. All aortic branches except for the ovarian artery were tied off as well as the vascular connections to the uterus and the oviduct. The ovarian veins were cannulated and the venous effluent collected. The concentration of microspheres in the venous effluent was determined in a Burkert chamber. In another group of animals microspheres were injected into the aortic blood stream and the venous blood from the ovary was microscopically examined.

The percentage of microspheres passed in the first group was in both follicular and in early and late luteal ovaries around 1%. All microspheres found in the venous effluent were 15μ or less in diameter. In the second group no microspheres were found in the venous effluent.

The results indicate that arterio-venous shunts alone in these types of ovaries cannot explain the above mentioned differences in blood flow rates obtained by the different measurement methods.

Increased oxygen extraction from hepatic arterial blood may compensate for the reduction in portal blood flow seen in hemorrhagic shock in cats (Krarup M Acta physiol scand 1972) The aim of the present study was to demonstrate to what extent a reduction in hepatic arterial blood flow may be compensated by increased oxygen extraction from portal venous blood.

In 8 splenectomized cats the hepatic artery was ligated after a control period and the following changes were observed portal venous flow increased 15% together with a slight decrease in portal venous pressure In 7 cats hepatic venous oxygen saturation decreased from 49 to 23% and hepatic oxygen consumption was unchanged Liver function as determined from the splanchnic elimination of ethanol hepatic uptake and excretion of Indocyanine Green and bile flow was not affected In 1 cat in which the control oxygen consumption of the liver was 60% higher than normal ligation caused an almost complete (92%) desaturation of the hepatic venous blood accompanied by a reduction in hepatic oxygen uptake and liver function and an increase in hepatic lactate output

It is concluded that when the hepatic oxygen consumption and portal blood flow are within normal limits a complete arrest of the hepatic arterial blood flow may be compensated by an increase in portal venous oxygen extraction

Investigations of the effects of drugs on major arteries from a number of lower vertebrates led Burnstock (Pharmacol Rev 1969 21 247) to conclude that the vasomotor innervation in fish is primarily cholinergic whilst adrenergic control increased during evolution toward higher vertebrates Adrenergic nerves in fish arteries have been demonstrated (Kirby S and G Burnstock Comp Biochem Physiol 1 1969 28 307)

In the present study the effect of cholinergic and adrenergic drugs on isolated strips from the celiac artery of the rainbow trout (Salmo gairdneri) and the cod (Gadus morhua) have been investigated Rainbow trout arteries are contracted by both noradrenaline ($pD_2=8.02$) and acetylcholine ($pD_2=6.62$) whilst the cod arteries are sensitive to noradrenaline ($pD_2=6.2$) but almost insensitive to acetylcholine (pD_2 less than 2.0)

The responses of the cod arteries to anticholinergics suggest the presence of adrenergic α -receptors since the order of potency of the catecholamines is adrenaline > noradrenaline > phenyl phrine and isoprenaline does not produce any contraction Furthermore the α -adrenergic blocking agent phentolamine and yohimbine competitively block the effect of noradrenaline on the preparation.

It is concluded that in the cod as compared to the rainbow trout cholinergic receptors and thus perhaps cholinergic innervation play little role in regulating the muscular tone of the celiac artery

- 161 Koefoed-Johnsen, V I Lyon and H.H Ussing (Institute of Biological Chemistry A University of Copenhagen, Denmark): EFFECT OF CU ION ON PERMEABILITY PROPERTIES OF ISOLATED FROG SKIN (RAHA TEMPORARIA)

Cu^{++} added to the external bathing solution of frog skin to a final conc of 10^{-4}M causes a marked decrease in chloride permeability and an increase in potential difference (Koefoed-Johnsen V and H H Ussing, Acta physiol scand 1958 42, 298) Chloride permeability decreases on an average to $1/4$ of control values in frogs which have been kept at room temp for a day or more We have now found that the effect of Cu^{++} is much less pronounced in skins from cold stored (at 5°C) frogs Cu^{++} increase the scm in cold adapted frogs an effect rarely seen in the warm adapted frogs

Sucrose and SO_4^{--} permeabilities are not affected by Cu^{++} treatment whether the frogs have been adapted to room temperature or not

The findings that sucrose and SO_4^{--} permeabilities are not affected by Cu^{++} while chloride permeability is considerably reduced seem to indicate two pathways for chloride ions 1) an intercellular shunt path which is not affected by Cu^{++} and 2) a cellular pathway which can be highly affected and reduced by Cu^{++}

- 162 Lang, L, Sjöberg E and C R Skoglund (Department of Physiology Karolinska Institutet, Stockholm Sweden): FROG SKIN GLAND ACTIVATION BY NERVE STIMULATION

Subcutaneous application of neurohormones has been a common way of activating frog skin glands whereas the effects of nerve stimulation have been less studied

In the present analysis a nerve-skin preparation from the calf, containing predominantly mucous glands was mounted so that the outside of the skin formed the bottom (0.63 cm^2 area) of a small test compartment of 0.5 ml volume filled with distilled water while the corneal side made contact with Ringer solution in a lower compartment

Glandular secretion was observed to occur at a stimulus strength of $3-5\text{ V}$ using pulses of 3 ms duration at 10 Hz ; the conduction speed of the sympathetic fibers involved was found to be $2-4\text{ m/s}$

Flame photometric determination of Na^+ and potentiometric analysis of Cl^- showed that optimal nerve stimulation at 10 Hz caused an increased outflow of Na^+ and Cl^- ions in equal amounts at a rate of about $1-2\text{ ug NaCl per cm}^2$ skin area per 10 s

The concomitant conductance changes occurring in the fluid of the test chamber were continuously monitored by using a technique previously described (Haapponen L and E R Skoglund Acta physiol scand 1967 69 51) This proved to be a convenient method of getting approximative information of the quantities and time relations of the glandular secretions in the course of an experiment thus allowing a direct comparison with skin potential changes

Frederiksen, O. (Inst. for Experimental Medicine, University of 163
Copenhagen, Denmark): EFFECT OF AMILORIDE ON THE TRANSEPITHE-
LIAL FLUID TRANSFER MECHANISM IN RABBIT GALL BLADDER IN VITRO

Amiloride (AM), a potent specific inhibitor of transcellular
Na transport in hypertonic transporting epithelia like toad
bladder, blocks Na-uptake and transfer completely within 1 min
when added to the mucosal side at concentrations of 10^{-5} - 10^{-4} M.
It has been claimed (Crabbé & Ehrlich, Pflügers Arch. 304:284,
1968) that AM is without effect on Na-transport in small in-
testine

In the present study the effect of AM on transcellular fluid
transfer in the isosmotic transporting rabbit gall bladder (GB)
was studied 'in vitro' using a gravimetric method. $0.88 \cdot 10^{-4}$
M was without effect. $0.88 \cdot 10^{-3}$ M produced a 60% reversible in-
hibition of mucosal-to-aerosol net fluid transfer when applied
from the mucosal side. It was without effect from the serosal
side. Thus isosmotic transfer by the GB is sensitive to AM but
to a smaller extent than hypertonic transporting epithelia.
 Ca^{2+} removal from the mucosal side did not significantly alter
the maximal response. This is contrary to findings in the toad
bladder (Cuthbert & Wong, Molec. Pharmacol. 8: 222, 1972). Pre-
liminary results indicate decreased O_2 -consumption with AM.
Furthermore, AM does not seem to alter total transepithelial
resistance. These findings suggest that the characteristics of
ion uptake and / or transcellular ion transport are different
in the two epithelial types

Nielsen, Robert (Institut of Biological Chemistry A, 164
University of Copenhagen, Denmark): EFFECT OF THE POLYENE
ANTIBIOTIC FILIPIN ON THE ACTIVE SODIUM TRANSPORT ACROSS
THE ISOLATED FROG SKIN

It has been shown previously that addition of the polyene
antibiotic amphotericin B to the medium bathing the outside
of the frog skin caused an initial small increase in the
short-circuit current (SCC) and the trans-epithelial poten-
tial (PD) followed by a great decrease in the PD (Nielsen R
Acta Physiol. scand. 1971 83: 106). Addition of filipin to
the outside caused qualitatively the same effects as amphi-
tericin B but the observed decrease in the PD was enhanced.
The addition of amphotericin B (5×10^{-5} M) to the inside had
no effect on the PD and the SCC. However the addition of
filipin (5×10^{-5} M) to the inside caused an increase in SCC
and PD. This increase of the SCC starts after about 5 min of
incubation with filipin. The activation has its first maximum
after 20-45 min of incubation thereafter the SCC starts to
decline to about the control level. After this decrease the
SCC increases again and reaches its second maximum after abo-
ut 2 hrs of incubation. The increase in SCC varied from 10-
100% of the control level.

It is concluded that filipin added to the inside of the frog
skin reacts at a site different from that it reacts with when
it is added from the outside

- 165 Sjöström M, Johansson R and Thornell L E (Dept of Anatomy, University of Umeå Sweden): THE STUDY OF PHYSIOLOGICAL ION FLUXES BY MICROANALYSIS OF ULTRATHIN SECTIONS OF FROZEN TISSUE

In ultrathin sections of untreated tissue prepared by cryo ultramicrotomy naturally occurring ions can be identified in situ by means of electron microscopical X-ray microanalysis. However in order for the data to be meaningful it is essential to define the functional state of the tissue at the time of freezing.

Between two pneumatically controlled chilled hammers is an oxygenated bath. In it a muscle is mounted between a micrometer screw and a tension transducer. The bath contains two parallel electrode plates for stimulation of the muscle. With the muscle stimulated or at rest the hammers are instantaneously brought together and at the same time the bath is drawn away. The muscle is frozen between the hammers. The muscle is in contact with air and thus unstimulated for less than 10 msec. After dry-sectioning the sections are freeze-dried.

Ultrastructure is well preserved which facilitates topographic orientation during analytical electron microscopy. As muscular processes are instantaneously interrupted in a defined state of contraction and as diffusible substances are assumed to remain in situ during the preparative procedure it should be possible to study physiological fluxes of ions on the ultrastructural level e.g. the flux of calcium during the contraction cycle. X-ray analyses of sections are in progress.

- 166 Wänge R and Åkerlund G (Department of Zoophysiology, University of Göteborg, Sweden): EFFECT OF ESERINE ON FLUID BALANCE IN MOLLUSCS

Certain drugs have been observed to disturb water balance in molluscs. Eggert and Umrath (Z vergl Physiol 1956 39 133) noticed that eserine causes swelling of freshwater gastropods. The mechanism of the effect is not known. We have investigated the effect of eserine and a few other drugs on a marine gastropod, the whelk (Buccina undatum). The molluscs were placed in bowls with sea water at 11°C. The drugs were dissolved in the sea water or in a few cases injected. Treated animals and controls were weighed at intervals. At the end of the experiments the approximate blood volume was estimated by bleeding. When whelks are treated with eserine or neostigmine at 10⁻⁵ - 10⁻³ g/ml the animals after 2-3 hours are highly swollen. The weight increase may be as high as 150 per cent of the initial body weight. Measurements of the blood volume show that eserine induces an enormous increase of the blood volume. Analyses of the blood showed no significant change of inorganic ions (sodium potassium calcium) but a marked decrease in plasma proteins. The osmotic pressure did not change significantly. The swelling was inhibited by sucrose at a conc. of 300 mM/kg in the surrounding sea water. The swelling seems to be secondary to muscle relaxation caused by the drugs.

Schofer, Almut and H - H Frey (Dept of Pharmacol and Toxicol School of Vet 167
Med Free Univ Berlin-West Germany): EFFECTS OF PROSTAGLANDINS E_2
AND $F_{2\alpha}$ ON BRONCHIAL TONE IN CATS

The study should provide dose-effect relationships for the 2 PGs and disclose possible interactions with the autonomic nervous system. Experiments were done in cats anesthetized with chloralose vagotomized completely relaxed by infusion of suxamethonium and artificially ventilated. Bronchoconstriction was induced and maintained by infusion of 5-HT, carbachol or histamine. Against 5-HT PGE_2 in the dose-range of 0.05-10 $\mu\text{g/kg}$ had a bronchodilator effect in all but 4 out of 22 cats in which the effect was constrictory. PGE_2 was less effective against carbachol-induced bronchoconstriction and ineffective against histamine. Dilator effects were slightly weakened by propranolol which in cats with normal bronchial tone intensified the constrictor effect. Reserpine pretreatment seemed to increase the sensitivity to the dilator effect; results with guanethidine and cocaine were variable and chlorisondamine and phentolamine did not modify the dilator effect of PGE_2 . This effect was dose-dependently intensified by indomethacin (2-10 mg/kg). - $PGF_{2\alpha}$ showed a rather weak bronchoconstrictor effect in the dose range of 0.1-50 $\mu\text{g/kg}$ which could not be modified by any of the above mentioned drugs. - The effects of the PGs on bronchial tone seem thus to be relatively independent of the autonomic nervous system but inhibition of PG synthetase by indomethacin resulted in an increased bronchodilator effect of PGE_2 .

Pjalland, B. (Department of Pharmacology and Toxicology H 168
Lundbeck & Co A/S Copenhagen Denmark): INHIBITION BY ANTI-INFLAMMATORY AGENTS OF THE RELEASE OF RABBIT AORTA CONTRACTING SUBSTANCE (RCS) AND PROSTAGLANDINS (PGs) FROM CHOPPED GUINEA-PIG LUNGS

It has been shown that PGs and RCS are released from lungs and other tissues by anaphylaxis and by mechanical agitation (Palmer et al Br J Pharmacol 1970 40 381P Gryglevski and Vane Br J Pharmacol 1972 45 37). In the present study the effect of some anti-inflammatory agents on the mechanically induced release of PGs and RCS from chopped unsensitized guinea-pig lungs has been investigated.

Stirring of the lung tissue resulted in release of activity corresponding to that of PGE_2 15 ng/ml as tested on the rat stomach strip. The PG-release was proportional to the time of mechanical agitation of the tissue.

Indomethacin when superfused over the lung tissue in a concentration of 20 ng/ml inhibited the release of PGs and RCS by about 50 % (IC_{50}). The IC_{50} for aspirin was about 2 $\mu\text{g/ml}$. Phenylbutazone was about 3 times as active as aspirin while sodium salicylate was inactive in concentrations up to 63 $\mu\text{g/ml}$.

The results suggest that inhibition of mechanically induced release of prostaglandins may be used as a simple in-vitro test for screening of anti-inflammatory agents.

169 Gustafsson L. P. Hedqvist and H. Lagercrantz (Dept of Physiology Karolinska Institute Stockholm Sweden): PROSTAGLANDIN MEDIATED ENHANCEMENT OF EFFECTOR RESPONSE TO CHOLINERGIC NERVE STIMULATION

Prostaglandin E_2 (PGE_2) has been shown to inhibit parasympathetic neuroeffector transmission in the rabbit heart (Wenmalm and Hedqvist Life Sci 1971 10 I 465). The present paper describes some PG actions in another cholinergically innervated tissue the bovine iris sphincter muscle.

Strips of the muscle were mounted in an organ bath with oxygenated Tyrode's solution at $37^\circ C$. Recordings of mechanical response were made isotonic field stimulation (1 msec 1-3/sec 5 sec/min) was shown to activate cholinergic nerves causing contraction of the muscle.

PGE_2 (0.2-10 ng/ml) potentiated contractions induced by transmural stimulation in a dose dependent manner. Higher doses increased resting base line and caused direct contraction. PGE_2 was equiactive with E_1 while PGF_2 was less effective in causing potentiation.

Administration of the PG synthesis inhibitor Eicosatetraenoic acid (ETA) markedly to completely depressed the response to transmural stimulation. Addition of a small dose of PGE_2 restored the response. ETA also removed the spontaneous and progressive increase in tone of the preparation normally terminating an experiment in 1-2 hours.

Prostaglandins are released from the bovine iris sphincter muscle (Pomeroy J. Brit J of Pharmacol vol 40 no 1 163P-164P). We suggest that endogenous prostaglandins may modulate smooth muscle response to parasympathetic nerve stimulation.

170 Stjärne, L. (Dpt of Physiology Karolinska Institutet Stockholm Sweden): MICHAELIS-MENTEN KINETICS OF CALCIUM-DEPENDENCE OF SYMPATHETIC NEUROTRANSMITTER SECRETION AND OF PROSTAGLANDIN DEPENDENT -INDEPENDENT FEEDBACK CONTROL OF THIS FUNCTION

In the isolated guinea-pig vas deferens the probability for (quantal) secretion of noradrenaline (NA) from sympathetic nerves on arrival of the nerve impulse seems to be restricted by two different control systems: one dependent and one independent of the local release of prostaglandin E_2 (PGE_2). The two systems seem to be additive in effect. Both are sensitive to changes in the NA concentration of tissue fluid; the NA level required to depress transmitter secretion by 50% is about 100 ng/ml in the absence of PGE_2 (local formation blocked by ETA) and about 50 ng/ml when PGE_2 synthesis is not blocked. The efficiency of both systems is inversely related to nerve stimulation frequency. Both mechanisms may operate by regulating the availability of calcium for the secretory mechanism. Application of Michaelis-Menten kinetic analysis seems to allow determination of K_m (5.2 mM calcium) and V_{max} (4.2×10^{-5} of the total store released per nerve stimulus) for the secretory mechanism. Exogenous PGE_2 (uncompetitively?) overcomes the disinhibition of NA secretion caused by α -blockade; PGE_2 not only depresses V_{max} but also raises K_m progressively more with falling calcium concentration in the medium. The potency of PGE_2 suggests that the PGE_2 -dependent control of NA secretion may operate a positive feedback loop (via (prejunctional?) α -adrenoceptors triggered by rising NA in tissue fluid) to depress the affinity for calcium of the secretory mechanism.

Kanje M Valum, E and Råström A (Dept of Zoophysiology 171
Göteborg Sweden): DIBUTYRYL CYCLIC AMP AND PROSTAGLANDIN E₁ INDUCE
MORPHOLOGICAL ALTERATIONS IN CULTURED HUMAN GLIOMA CELLS
Neuroblastoma cells (C 1300) treated with dibutyryl cyclic AMP
(dBo-AMP) or prostaglandin E₁ (PGE₁) differentiate within 24 h
Protein- but not RNA-synthesis is necessary for this differentiation
which fails if the integrity of the microtubular system is destroyed
(Prasad K and Vornadakis A Exp Cell Res 1972 70 27 ; Prasad
K Nature New Biol 1972 236 49) Hamster ovary cells (CHO-K1)
treated with the same drugs rapidly (1 h) change their morphology and
become density dependent inhibited This effect is independent of
protein- as well as RNA-synthesis (Hsie A and Puck T Proc natl
Acad sci 1971 68 358)

Cultured glioma cells (138 MG) normally have an irregular flattened
shape We report here that 138 MG like C 1300 and CHO-K1 cells undergo
morphological changes in the presence of dBo-AMP and PGE₁ Within one
hour the glioma cells elaborate up to 150 μ long multiple processes
The alterations were accentuated in serum-free medium Morphological
changes did not take place in the presence of vinblastine whereas
they were unaffected by actinomycin D (10 μ g/ml) as well as cyclo-
heximide (40 μ g/ml)

The vinblastine effect suggest that microtubuli are involved in
the morphological changes of glioma cells The dBo-AMP and PGE₁ indu-
ced changes appeared more rapidly showed reversibility and were in
contrast to the effects on C 1300 not dependent on de novo protein-
-synthesis In the latter respects 138 MG resemble CHO-K1 cells

Ekblom M, Lindholmer C & Johnsen M (Reproductive Physiology Unit 172
Dept Physiology Karolinska institutet S-104 01 Stockholm Sweden):
EFFECTS OF HUMAN SEMINAL PLASMA ON SOME FUNCTIONAL PROPERTIES OF THE HUMAN
SPERMATOZOA.

The seminal plasma has a remarkably complex composition indicating func-
tions beyond that of providing nutriment for the spermatozoa during their
short contact with the plasma and a vehicle for the transportation of
spermatozoa into the female genital tract

Various functional properties of human spermatozoa have now been studied
in 1) whole semen 2) various fractions of "split-ejaculates" (the first
part of the human ejaculate contains mainly prostatic fluid the last por-
tion mainly fluid from the seminal vesicles) and 3) buffered salt solutions

The oxygen consumption of the spermatozoa was significantly higher in salt
solutions than in whole semen The vesicular fluid had negative effects on
sperm motility and survival and caused an increased uptake of zinc by the
spermatozoa The prostatic fluid had a motility promoting effect on washed
spermatozoa and protected the spermatozoa from the adverse actions exerted
by the vesicular fluid

A better understanding of the functional relationship between the male
accessory genital glands and the spermatozoa is of importance from different
points of view Identification of functional parameters important to the
fertility of human spermatozoa and knowledge how such parameters can be
influenced by endogenous and exogenous factors secreted by the accessory
genital glands can give leads to new reversible male contraceptive agents
that does not interfere with the spermatogenesis

- 173 Persson N A and Hedqvist, P (Department of Physiology I Karolinska Institute Stockholm Sweden): REDUCED INTESTINAL MUSCULAR RESPONSE TO ADRENERGIC NERVE STIMULATION AFTER THE ADMINISTRATION OF PROSTAGLANDINS

Prostaglandins E (PGEs) inhibit sympathetic neuroeffector transmission in organs such as the spleen heart and vas deferens. This paper describes similar effects produced by PGE₁ and PGE₂ on effector responses to adrenergic nerve activity in the rabbit intestine.

Pieces of rabbit jejunum and ileum with intact adrenergic nerve supply were mounted in an organ bath and kept in oxygenated Tyrode at 37°C. The adrenergic nerves were stimulated with square wave pulses (3-10 Hz, 1 msec duration, supramaximal voltage) and all recordings of mechanical activity were made isotonically (load 1-2 g).

In the absence of treatment the preparation showed spontaneous and rhythmic contractions. Stimulation of the adrenergic nerves or administration of NA caused a graded inhibition of the intestinal movements. Low doses of PGE₁ and PGE₂ (1-12 ng/ml) which *per se* showed little or no effect on the spontaneous contractions of the preparation consistently antagonized the inhibitory action of adrenergic nerve stimulation on intestinal motility in a dose-dependent manner. On the other hand, the inhibitory action of NA was little affected by the PGEs. It is therefore suggested that the PGEs inhibit adrenergic responses in the intestine by reducing the release of NA from the adrenergic nerve terminals.

- 174 USE OF CELL CULTURE TO STUDY BIOCHEMICAL, PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF ISOLATED NEURONS AND GLIAL CELLS. L. Hertz and L. Dittmann - of Biochemistry A University of Copenhagen DK-2100 Copenhagen and M Sensenbrenner and F Mandel Centre de Neurochimie du CNRS Strasbourg France

- Determination of metabolic events in the different cell types of the brain is encumbered with considerable uncertainty due to the complexity of the tissue. Cell cultivation provides means either to obtain glial cells and neurons separately and without mutual contamination in amounts which are large enough (50-100 cells) to determine respiration by aid of Cartesian microdivers or to obtain cultures consisting of respectively pure glial cells and a mixed population of neurons and glial cells. These cultures can be compared in flux studies. The rate of oxygen uptake by the cultivated glial cell is relatively high (130 μ moles/g wet wt/h) though not as high as that by the neurons (570 μ moles/g wet wt/h). These values compare favorably with the respiratory activity in brain slices and in the brain *in vivo*. An increase of the potassium concentration causes a significant rise of the oxygen consumption in the glial cells but has no effect on the neurons (cf Hertz J Neurochem. 1968 13 1373). Flux measurements (Latzkovits Sensenbrenner & Mandel in preparation) indicate a glial-neuronal interrelationship with respect to potassium transport. Taken together the results emphasize the role of glial cells in brain physiology and biochemistry. Pharmacological studies of the two cell types are in progress.

In 197 we could report that activity evoked by electrical and adequate stimuli and mediated via ventral spinal afferent pathways will give a cortical response in young kittens most marked at the age of one to three weeks and after this age decreasing in size. We have now examined the thalamic representation for this pathway in 30 kittens using microelectrode technique and identifying the recording site by physiological as well as histological methods.

Short latency potentials were most evident in the region of nucleus centrum medianum sometimes with a high degree of convergence. There were no responses from ventral pathways obtained in VPL. However some potentials with longer latency were found anterior and posterior to this nucleus.

Taylor T (+) and Skrede, E K (Institute of Neurophysiology University of Oslo Norway) PRESERVATION OF SYNAPTIC MECHANISMS IN ISOLATED SLICES OF MAMMALIAN HIPPOCAMPAL CORTEX

A central question in the use of the slice technique regards the possibility of electrophysiological abnormality due to tissue damage.

To study whether slices can show physiological responses comparable to those in intact preparations we used slices cut transversally to the longitudinal axis of the hippocampus in which the major pathways can be preserved due to its lamellar organization (Andersen, Blaes & Skrede Exp Brain Res 1971 13 222-238). Such preparation contain a four-membered neuronal loop with three well-defined excitatory synapses (Skrede & Westgaard Brain Res 1971 35 589-593). The present study was intended to investigate synaptic facilitation and inhibition in one link of this loop the connection between the CA3 and CA1 pyramids.

Stimulation of these fibres and the alveus respectively gave orthodromic and antidromic population potentials of CA1 pyramids similar to those in vivo. The CA1 response to repetitive orthodromic activation was a pronounced frequency-dependent facilitation. Its time course was examined using double shock techniques and found to be comparable to that of the intact preparation. The phenomenon could be elicited without population spikes being recorded from any part of the CA1 area suggesting a non-recurrent mechanism. A powerful inhibitory mechanism was also demonstrated which in contrast proved to be recurrent.

We conclude that the described inhibition and facilitation is of the same nature as demonstrated in vivo.

(+) Presently at Psychology Dept Harvard Univ Cambridge Mass U.S.A

- 177 Hoff B Ungerstedt U and Siggins E (Dept of Histology Karolinska Institutet Stockholm Sweden): ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES ON CAUDATE NUCLEUS NEURONS AFTER DESTRUCTION OF DOPAMINE CONTAINING AFFERENTS

The effects of destruction of dopamine-containing afferents on the physiological and pharmacological properties of caudate neurons was studied in halothane-anesthetized rats. Action potentials from single neurons were recorded with multibarrel micropipettes and drugs were administered at the site of recording using microiontophoresis. One week prior to recording 6-hydroxydopamine was injected unilaterally into the substantia nigra to eliminate the dopamine projection to the ipsilateral caudate. In normal caudate the discharge of neurons is reproducibly and uniformly reduced by local application of dopamine or apomorphine. After destruction of dopamine-containing afferents by 6-hydroxydopamine the mean threshold iontophoretic current for dopamine and apomorphine inhibition are significantly reduced. Moreover the spontaneous discharge of caudate neurons is markedly elevated after elimination of dopamine input. Normal caudate neurons usually discharge spontaneously at less than 0.5/sec or are silent. After 6-hydroxydopamine most caudate units fire at rates of 2.5-5/sec; a significant proportion discharge at rates greater than 10/sec. These results suggest (1) that apomorphine mimics the effects of dopamine on caudate neurons (2) that apomorphine-induced inhibition is not mediated via dopamine-containing terminals (3) that supersensitivity to the electrophysiological effects of dopamine and apomorphine develops after destruction of dopamine-containing afferents (4) that the dopamine pathway exerts a tonic inhibitory influence on caudate neurons.

- 178 PLASTICITY OF CENTRAL NORADRENALINE (NA) NEURONS FOLLOWING ADMINISTRATION OF 6-HYDROXYDOPAMINE (6-OH DA) TO NEWBORN RATS Chris Pycock, Charlotte Sachs and Gösta Jonsson, Dept. of Physiology Karolinska Institutet, Stockholm.

Unilateral injection of 6-OH DA (3×100 mg/kg s.c.) to newborn rats was found to cause a selective and permanent reduction in endogenous NA and uptake in the forebrain, being about 30% of control when analysed at the adult stage. However, the NA concentration and 3H NA uptake in the pons medulla region was increased by 75%. Analysis of 3H NA uptake kinetics showed similar K_m values for 6-OH DA treated and control rats. Subcellular distribution studies on endogenous NA in the pons medulla showed the highest relative NA increase in the microsomal fraction considered to contain the amine storage granules. The distribution pattern of NA synaptosomes from the pons medulla loaded with 3H NA and centrifuged on a continuous sucrose gradient differed between control and 6-OH DA treated rats, the peaks being at a sucrose concentration of 1.1 M and 1.25 M respectively. Fluorescence histochemistry displayed a reduced number of nerve terminals in the cerebral cortex, whereas in the pons medulla an increased number of nerve terminals with an increased fluorescence intensity was seen. The present results show that 6-OH DA administered at birth results in a selective degeneration of NA nerve terminals in the forebrain, while in the pons medulla this treatment leads to an increase in NA concentration in the nerve terminals and possibly also a growth of NA terminals, probably related to the damage of the terminals in the forebrain.

Skauhen, E. (Departments of Physics and Physiology University 179
of Oslo, Norway) POSSIBLE FUNCTIONAL SIGNIFICANCE OF THE GEO-
METRY OF THE INITIAL SEGMENT IN NERVE CELLS

The density of Na^+ conducting pores in nerve membranes is rather low (Colquhoun D et al J. Physiol 227 95) The opening and shutting of the pores is a random process and the membrane potential will therefore tend to fluctuate. The fluctuations may be noticeable and important in regions of nerve cells where the diameter and thus the area of the nerve membrane is small.

Simulation of nerve cells with a uniform distribution of pores with equal properties has been performed on a digital computer. The random process was governed by equations of the Hodgkin Huxley type. It was found that the action potential started where the axon had the smallest diameter (at the initial segment). When this diameter was decreased the firing frequency increased. The relative increase was largest at small values of the injected current.

It is concluded that a small diameter of the initial segment facilitates the initiation of the action potential at this place and makes the firing frequency a more linear function of the injected current.

Røed, A. (Institute of Physiology and Biochemistry, Dental 180
Faculty University of Oslo, Norway) HIGH FREQUENCY INHIBI-
TION OF THE SARCOLEMA BY PROPRANOLOL

Propranolol has been shown to produce a high frequency inhibition of the rat phrenic nerve-diaphragm preparation during direct stimulation. Twitch contractions were unaffected (Lilleheill G and Røed A Arch int Pharmacodyn 1971 194 129). In the present experiments the mode of action of propranolol on the sarcolemma was studied.

Propranolol, 2×10^{-6} g/ml did not affect low frequency muscle action potentials. During 50/sec stimulation the action potentials appeared as an initial train with gradual development of initiation delay, reduced steepness of rising and falling phases and amplitude reduction. The train disappeared and was followed by irregularly appearing action potentials. Greater inhibition was characterized by a shorter and less distorted train and reduced or abolished irregular activity. Trains never reappeared during continued stimulation but could again be evoked after a short interruption of stimulation. Accordingly the effect of propranolol is caused by an interference with stimulus excitation coupling and not by a prolonged refractory period. An elevation of threshold was probably involved since the inhibition could be reduced by increasing the stimulating current.

- 181 Leppäluoto J, Lybeck, H and Virkkunen P (Institute of Physiology, University of Helsinki Finland) INHIBITION BY HUMAN PLASMA OF THE BIOLOGICAL ACTIVITY OF THYROTROPIN RELEASING HORMONE (TRH)

It has been shown that the biological activity of TRH is destroyed when the hormone is incubated with plasma. Inactivation is believed to occur by splitting a part from TRH molecule or by binding TRH with plasma proteins (Bassiri R and Utiger, R. *Endocrinology* 1972 91 657). We have studied the binding of TRH to plasma proteins and the capacity of various plasma fractions to inactivate TRH.

TRH (Ferring Ltd) was added to plasma submitted to gel filtration (Sephadex G-200). The plasma protein fractions were bioassayed for TRH in mice pretreated with T-3 and I 131 and they showed no TRH-activity. Untreated plasma was then separated into 4 fractions by gel filtration and the fractions were incubated with TRH for 90 min. TRH was inactivated by the fraction with the relative elution volume of 1.6 - 1.8. We conclude that TRH is inactivated by a factor in plasma, possibly a protein enzyme.

- 182 HELGASON S ET (Department of Zoophysiology University of Göteborg Sweden, and Department of Physiology, University of Iceland): EFFECTS OF CALCITONIN ON INDUCED HYPERCALCAEMIA IN RAINBOW-TROUT *SALMO GAIRDNERI*

Parathyroid glands are absent in fish but ultimobranchial cells secrete calcitonin. In some Teleosts calcitonin acts the calcemia in others, i.e. Salmonids calcitonin

has been reported to be without effect on plasma calcium. Most fishes are hypercalcemic with regard to their natural environment be it fresh water or sea water. In the present experiment a severe calcium load was inflicted on 19 rainbow-trouts by adding CaCl_2 to fresh water to final concentration of 1 g/100 ml. Another group of 10 fish was kept in fresh water. Both groups received 40 mU calcitonin/100 g, and the calcemia was measured at t 0 and t 6 hours after calcitonin administration. The fresh water group showed no changes in plasma calcium 6 hours after the injection. Half of the fish kept in calcium rich water for one week had become strongly hypercalcemic and calcitonin injection decreased plasma calcium to normal values in 6 hours. The remaining 5 fish, kept in calcium rich water, did not exhibit hypercalcemia after 7 days and calcitonin injection was without effect on plasma calcium.

It is suggested that endogenous secretion of calcitonin in calcium treated fish which did not respond to exogenous calcitonin is sufficient to prevent hypercalcemia to develop. CHAN D K O et al. *Gen comp Endocr* 1958 11 243-245. PANG P K T, *J Exp Zool* 1971 178 89-100.

Russell J T and Thorn N A (Institute of Medical Physiology C University of Copenhagen, Denmark): EFFECTS OF INHIBITORS OF CALCIUM TRANSPORT (D600 AND PRENYLAMINE) ON RELEASE OF VASOPRESSIN IN VITRO 183

The presence of Ca^{++} is obligatory for the release of vasopressin from neurohypophyses in vitro and calcium seems to be taken up during the stimulation. We have examined the effect on vasopressin release of D600 (a Verapamil derivative) and prenylamine which both inhibit calcium transport in smooth and cardiac muscle. Halved rat neurohypophyses were incubated in a bicarbonate buffered medium with 2.8 mM Ca^{++} . Stimulation of secretion was carried out by the application of a field current or with 56 mM K^+ containing medium. D600 (4, 10, 20 μ M) and prenylamine (20 and 100 μ M) produced a dose related inhibition of vasopressin release in both stimulation types. D600 also inhibited the increased release caused by the introduction of Ca^{++} into a Ca^{++} free medium during electrical stimulation. However prenylamine did not inhibit this release.

The results suggest that an enhanced Ca^{++} transport over the cell membrane (which can be blocked by D600) precedes hormone release and that prenylamine acts at a subsequent step in the stimulation secretion coupling.

Vilhardt H & Hope D E (Department of Pharmacology University of Oxford, O X 2); DEMONSTRATION ON POLYACRYLAMIDE GELS OF ATPases FROM BOVINE NEUROHYPOPHYSES 184

Previous investigations (Poisner A M & Douglas W W, Mol Pharmacol 1968 4 531; Vilhardt H & Holmer G Acta endocr (Kbh) 1972 71 538) have demonstrated ATPase activity in homogenates and subcellular fractions of mammalian posterior pituitary glands.

In the present experiments the ATPase activity of subcellular fractions of bovine neural lobes was studied. Three types of activity were found: Mg^{++} -, Ca^{++} - and Mg^{++} + Na^+ + K^+ activated. The ratio between the 3 enzymes varied between the subcellular fractions. The enzymes were all membrane-bound but the Mg^{++} - and Ca^{++} -ATPase could be solubilized by 1 % Triton X-100 with only minor loss of activity. The activity of the Mg^{++} + Na^+ + K^+ -ATPase was completely inhibited by this concentration of Triton X-100.

The Triton X-100 solubilized proteins were subjected to electrophoresis on 5 % polyacrylamide gels. The position of each ATPase in the gels was demonstrated by incubating the gels with ATP and either Mg^{++} or Ca^{++} followed by the addition of Pb^{++} . The P_i liberated from ATP by the enzymes was precipitated as white bands in the gels. In this way it could be shown that while the Mg^{++} -ATPase was also activated by Ca^{++} the Ca^{++} -ATPase seemed not affected by Mg^{++} .

185 Møller, W. K. Møllgård & E. C. Sørensen (Anatomy Department A & Institute of Medical Physiology Dept. A University of Copenhagen Denmark): CHANGES IN THE ULTRASTRUCTURE OF THE CAROTID BODIES FOLLOWING PROLONGED EXPOSURE TO HYPOXIA

The carotid bodies are considered primarily to be chemoreceptors in the ventilatory reflex response to changes in arterial pO_2 , pCO_2 and pH but their ultrastructure suggests that they might be organs of internal secretion. This study was performed in order to find out if their ultrastructure is changed following prolonged exposure to hypoxia. Four rabbits were obtained in La Paz, Bolivia (altitude 3800 m). They were bred and had lived at an altitude of 4000-4300 m. Another four rabbits were exposed to hypoxia equivalent to an altitude of 6000 m in a low pressure chamber for seven days. The carotid bodies were fixed by perfusion in vivo with glutaraldehyde, postfixed with OsO_4 , uranyl blue-stained and embedded in Epon (R). In both groups we found a marked increase in the number of electron dense core vesicles and mitochondria in the type I cells compared to sea level controls.

This finding is compatible with an increased production of an at the present time unknown substance in the type I cells during prolonged hypoxia.

Meland, L., Kinemuchi, H. and Yoo, B. Y. (Department of Pharmacy, University of Umeå, Sweden): THE MECHANISM OF ACTION OF THE MONOAMINE OXIDASE INHIBITOR PARGYLINE

Pig liver monoamine oxidase has previously been shown to contain one mole of covalently bound FAD per mole of enzyme. It has also been shown that by inhibition with the irreversible inhibitor N-methyl-N-2-(propynyl)-benzylamine (Pargyline) the inhibitor is bound to the enzyme in an equimolar ratio to flavin. In order to investigate the nature of the binding of Pargyline to the active site, purified pig liver monoamine oxidase was inhibited by ^{14}C -labelled Pargyline and then extensively digested by pronase. The Pargyline-containing fragment was purified by gel filtration, ionic exchange chromatography and thin layer chromatography. The equimolar ratio between Pargyline and flavin was shown to be retained after the purification procedure. Thin-layer chromatography in several systems with subsequent localization of flavin in UV-light and Pargyline by autoradiography has further indicated that Pargyline is bound to the flavo-peptide. Amino acid analyses of the Pargyline-containing flavo-peptide after performic acid oxidation yielded cysteic acid, aspartic acid, serine and glycine in a molar ratio of 1:1:1:2.

Hansen A J & S C Sørensen (Institute of Medical Physiology 187
Dept A University of Copenhagen Denmark) CHANGES IN THE LDH

ISOENZYME PATTERN IN RABBIT BRAINS FOLLOWING PROLONGED HYPOXIA
In most tissues there are five LDH-isoenzymes each composed
of different proportions of H and M subunits. It has been shown
in various tissues in vivo and in vitro that the relative con-
centration of H-subunits decreases when PO_2 is lowered. This is
assumed to reflect a greater anaerobic glycolysis in the tissue.
We have compared the LDH-isoenzyme pattern in cortical grey
matter corpus callosum cerebellar cortex and inferior colli-
culi from rabbits exposed to hypoxia equivalent to an altitude
of 6000 m for seven days with that in rabbits living at sea
level. The LDH-isoenzymes in supernatants from homogenates were
fractionated by Agarose gel electrophoresis and the relative
concentrations of H subunits were calculated from the scanning
curves. The concentration of H subunits (mean \pm SD) in six
hypoxic rabbits and six sea level rabbits respectively were:
cortical grey matter: 71 \pm 0.8 and 70 \pm 1.3; corpus callosum:
74 \pm 1.7 and 76 \pm 1.4; cerebellar cortex: 81 \pm 0.9 and 73 \pm 1.2;
inferior colliculi: 89 \pm 0.8 and 81 \pm 0.6. The differences are
only significant in cerebellar cortex and inferior colliculi
($p < 0.01$). Thus there is regional differences in the changes in
the LDH-isoenzyme pattern. However the results suggest that in
some areas of the brain the degree of anaerobic glycolysis is
increased during prolonged hypoxia which might be of importan-
ce for the ventilatory acclimatization to chronic hypoxia.

Oreland, L and Kijimauchi H (Department of Pharmacology
University of Umeå Sweden): THE SUBUNIT STRUCTURE OF PIG
LIVER MITOCHONDRIAL MONOAMINE OXIDASE (MAO)

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The molecular weight of pig liver MAO has previously been
shown to be about 115 000 with one mole of covalently bound
FAD per mole of enzyme. Gel filtration of purified enzyme on
Sephacel 4 B in 6 M guanidine and 0.1 M mercaptoethanol (MCE)
and analytical ultracentrifugation in 0.1 % sodium dodecyl sul-
phate (SDS) and 0.1 % MCE yielded molecular weights of 55 000
and 63 000 respectively. By polyacrylamide gel electrophore-
sis in 0.1 % SDS + MCE one band of 62 000 mwt appeared. These
results seems to imply that the enzyme is composed of two sub-
units of which one carries the active site. If MCE was omitted
during the gel electrophoresis two equally large bands of
about 60 000 mwt were formed. By using enzyme inhibited by ^{14}C -
Pargyline a MAO-inhibitor blocking the active site of the en-
zyme in a 1:1 molar ratio it was found however that both
bands contained Pargyline in a ratio of 1 mole per 140 000 g
of protein. Furthermore amino acid analyses yielded the same
amino acid composition of the two bands. The results are inter-
preted that the enzyme is composed of two subunits of identical
molecular weight (about 60 000) of which only one contains the
active site and that the enzyme preparation contained two forms
of the enzyme presumably differing in the state of oxidation
of SH-groups.

189 Herlitz, H. and R. Hultborn (Department of Physiology and Institute of Neurobiology University of Göteborg, Sweden): ADVANCES IN A SPECTROPHOTOMETRIC PROCEDURE FOR MEASUREMENTS OF OXYGEN CONSUMPTION IN MICRO-SCALE.

A microspectrophotometric procedure for determination of respiration of minute tissue samples has been developed as an alternative to existing micromanometric techniques (Herlitz H. and Hultborn, R. *Acta physiol. scand.* 1973 87 4A; Hultborn, R. *Anal. Biochem.* 1973 47 442). The tissue sample is incubated together with an oxyhemoglobin solution within an air-tight glass-chamber and the absorbance change of the solution is recorded spectrophotometrically when the oxyhemoglobin is deoxygenized. The sensitivity can be varied from 10^{-6} - $1 \mu\text{l O}_2/\text{hr}$ by changing the volume of the incubating chamber. In the present study samples of rat corpus luteum tissue were assayed for oxygen consumption in absence and presence of succinate using chambers drilled in object slides giving a volume of approx. $1 \mu\text{l}$ and a sensitivity in the order of $10^{-2} \mu\text{l O}_2/\text{hr}$. This system has been characterized by our group (Herlitz, H. and Hultborn, R. *Acta physiol. scand.* 1973 87 4A) and the methodological results can thus easily be evaluated. Three major improvements have been introduced since the former report (Herlitz, H. and Hultborn, R. *Acta physiol. scand.* 1973 87 4A): 1. An automatic cuvet changing device has been developed increasing the measuring capacity 6-fold enabling up to 24 determinations a day. 2. Reagents can be introduced into the chamber during the experiment so that metabolic effects can be studied using the same sample of tissue thus minimizing biological and experimental variation. 3. O_2 -affinity of hemoglobin is decreased using inositol hexaphosphate thus increasing the oxygen tension at which recordings of respiration are performed.

190 Edman K.A.P. and M. Johansson (Department of Pharmacology University of S-223 62 Lund Sweden): THE CONTRACTILE STATE OF RABBIT PAPILLARY IN RELATION TO STIMULATION FREQUENCY

There is evidence (Koch-Jeser and Blinks *Pharmacol. Rev.* 1963 15 601.) that the cardiac action potential in addition to being a trigger of the mechanical activity exerts both a positive and a negative effect on the subsequent contractile state of the muscle. The aim of the present study has been to further elucidate these two effects. Rabbit papillary muscles (37°C) were stimulated by passing rectangular DC pulses (2 ms duration) through platinum plate electrodes. The stimulation frequency was varied between 0.3 and 420 per min. AC stimulation (20 Hz) was also used. The conventional force-frequency curve was determined from measurements of the steady-state twitch amplitude at different stimulation rates. The curve exhibited a maximum at 200-300 stimuli per min. The twitch amplitude in response to a test stimulus applied at different times after a stimulation period was studied. It was deduced from such measurements that the negative effect of excitation disappeared with a time constant of 150 ms whereas the positive effect of excitation declined with a time constant of approx. 100 s. The twitch amplitude recorded 1 s after a preceding stimulation period was used as an index of the maximal contractile state of the muscle (MCS) at the stimulation frequency considered. MCS increased steadily with the stimulation frequency. The highest value of MCS was recorded after a period of 20 Hz AC stimulation, this value being 2.5 times greater than the steady state twitch tension recorded at optimal frequency. An increase in the external calcium concentration from 2 mM to 8 mM had little effect on the peak value of MCS.

Harri M, M E, R Tirri and L Laitinen (Zoophysiological 191 Laboratory, Department of Zoology, University of Turku, Finland): LOWERED CHRONOTROPIC SENSITIVITY OF RAT AND FROG HEARTS TO SYMPATHOMIMETIC AMINES FOLLOWING COLD ACCLIMATION

Cold acclimation is known to increase the metabolic sensitivity to sympathomimetic amines in mammals (Carlson, L D, *Pharmacol. Rev* 1966 18 490) as well as in amphibians (Harri, M. and R. Hedenström, *Comp Biochem Physiol* 1972 41A 409). Thus, the effect of cold acclimation on the chronotropic sensitivity of the heart was studied.

The test drugs were injected intravenously to anesthetized rats and to pithed frogs *Rana temporaria*.

- Cold acclimation elevated the heart rate in the rat and decreased the chronotropic sensitivity to noradrenaline and isoprenaline. These changes were temporary. Phenylephrine failed to increase the heart rate. In the frog, cold acclimation lowered the chronotropic sensitivity of the heart most to phenylephrine, less to adrenaline and not at all to isoprenaline, as indicated by changes in the ED₅₀-values. In addition, the elevation of the measuring temperature decreased the sensitivity to phenylephrine in this animal.

The results obtained are suggested to be explained by decreased receptor sensitivity and increased metabolic rate and CORT activity in the heart tissue caused by increased sympathetic activity at low temperatures.

Kajise, L. L A Carlson, B W Larsson H Löw M Wahlqvist (Department 197 of Clinical Physiology and Endocrinology and King Gustaf V Research Institute Karolinska sjukhuset Stockholm and Department of Geriatrics Uppsala University Sweden): SUBSTRATE AND HORMONE CONCENTRATIONS IN THE REGULATION OF SUBSTRATE UPTAKE BY THE HUMAN HEART

The myocardial extraction of a substrate is mainly determined by the blood concentrations of substrates and hormones. We have studied the relative importance for the extraction of a substrate of its own blood concentration, concentrations of other substrates and hormones. In normal man by coronary sinus catheterisation both in the fasting state after lowering plasma free fatty acid (FFA) concentrations by nicotinic acid and increasing substrate concentrations by the infusion of glucose and a fat emulsion.

Myocardial extractions of glucose, FFA and lactate were correlated with their arterial concentrations. Glucose extraction was not directly correlated with insulin concentration but multiple regression analysis revealed a significant correlation with insulin as well as growth hormone, glucocorticoid and FFA. Assuming only one independent variable was altered at a time a 10 % increase in arterial concentration of either insulin, growth hormone, glucocorticoid or FFA would result in respectively 25 % increase, 6 % decrease, 11 % decrease or 10 % decrease in myocardial glucose extraction. Exogenous fat in the form of chylomicrons seemed to exert the same negative effect on glucose extraction as did FFA.

193 Jlebekk A and Lekven J (Institute for Experimental Medical Research University of Oslo Ullevål Hospital Norway) EFFECTS OF NICOTINE ON CARDIAC MECHANICS

Nicotine increases ventricular stroke volume (SV) but it is not known whether this effect is a consequence of increased end-diastolic volume or increased myocardial shortening during the ejection period. To examine this problem ventricular dimensions and fiber shortening (FS) were measured by ultrasound technique (Bugge-Asperheim et al. Scand J clin Lab Invest 1969 24, 361) on anesthetized open-chest dogs.

Intravenous infusion of nicotine (20 µg/kg min) raised SV by $25 \pm 7\%$ and left ventricular systolic pressure (LVSP) by 37 ± 6 mm Hg. Both FS and end-diastolic dimensions rose ($p < 0.01$).

When LVSP was raised by aortic constriction to the same value as during nicotine infusion SV was slightly reduced. Myocardial contractility and FS were also slightly reduced but end-diastolic dimensions increased more than after nicotine ($p < 0.05$).

It is concluded that increments in both FS and end-diastolic ventricular dimensions contribute to the increase in SV. Since end-diastolic dimension rose more during aortic constriction the increased FS represent the predominating cardiac mechanical event leading to increased SV under the influence of nicotine.

194 Haglund U, O Isaksson and O Lundgren (Dept of Physiology University of Göteborg Sweden): CARDIAC EFFECTS OF BLOOD BORNE MATERIAL RELEASED FROM THE SMALL INTESTINE IN SHOCK

A pronounced cardiovascular derangement is often observed in the cat after a two hour period of a simulated shock situation in the small intestine (regional hypotension at 30 mm Hg during occlusion of the nervous vasoconstrictor fibres). It

is proposed that these effects were caused by blood borne cardio-depressant substance(s) released from the "shocked" small intestine (Haglund and Lundgren, *Acta physiol scand* In press). In order to obtain further evidence for this hypothesis a study on an *in vitro* heart preparation has been performed. Rat hearts were perfused anterogradely according to the technique of Morgan et al (*Fed Proc* 1965 24: 1040). By this technique both the aorta and left atrium are cannulated and the heart is performing an actual mechanical work. 40 ml of oxygenated Krebs-Henseleit bicarbonate buffer containing glucose was recirculated and the left ventricular pressure was continuously monitored by a pressure transducer. After an initial perfusion period to stabilize the heart function 2-4 ml of venous plasma from control or shocked intestinal segments was added to the perfusion medium. In 9 of 10 experiments shock plasma induced a pronounced depression of cardiac function within 2-8 min as revealed by a decrease in peak systolic pressure. Control plasma caused no such effects. Thus the present results strongly suggest that cardio-depressant material is released from the small intestine in shock. Moreover this heart preparation seems to be a convenient bioassay system in evaluating what kind of substance(s) that induce the cardiac hypofunction in shock.

Using pulsed reflected ultrasound the displacement $s(t)$ of an easily recognized area of the posterior left ventricular wall can be recorded. On line differentiators were developed to differentiate the $s(t)$ curves hereby providing the velocity ($V = ds/dt$) and acceleration ($\dot{A} = d^2s/dt^2$) of the epicardial movement observed. The standardized technique was evaluated in six healthy young men with phonocardiography and stroke volume determinations (CO_2 rebreathing method) both at rest and during supine cycling (150-300-450-600-750-900 kpm/min). The positive peak values of the first and second time derivatives of the $s(t)$ curve were located almost constant relative to the auscultatory systole at all exercise levels and the period of positive velocity (CED-systole) constituted approximately 90% of the auscultatory systole being equivalent to the ejection period at least at rest. The peak displacement (Δs) increased linearly with stroke volume and adjustment for individual heart volume showed that stroke volume was proportional to the arbitrary variable (Δs heart volume). The four subjects with the largest maximal oxygen uptake per weight unit had the largest increment in positive peak velocity (\dot{V}) per heart rate increment indicating that the fittest subjects had a higher contractility increment per unit of tensile stress. The CED examination is practicable even during heavy exercise and might yield physiological information concerning alterations in stroke volume and myocardial contractility on a beat to beat basis.

The force-velocity (P-V) relation in rabbit papillary muscle was analyzed at various temperatures (23-32 °C) using the damped-release method described previously (Edman and Nilsson Acta physiol scand 1972 85 488). A lowering of the temperature induced a considerable increase in P_0 (the tension at zero velocity) whereas V_{max} (the shortening velocity at zero load) was only slightly affected. This is in contrast to the effects produced by various inotropic interventions (cardiac glycosides, adrenaline, calcium and altered stimulation frequency) in which cases P_0 and V_{max} are affected to very nearly the same degree. The temperature-induced changes of the P-V curve were interpreted as being the net result of two opposing effects: 1) an altered degree of activation of the contractile system caused by a change in the concentration of activator-calcium at the contractile sites (inotropic temperature effect) and 2) a change in the rate of interaction between the A- and I-filaments (specific temperature effect). The latter effect would be reflected by the change in V_{max} after correction for the inotropic effect. For the purposes of the present analysis the change in P_0 was used as a measure of the inotropic temperature effect. By allowing for an equally large inotropic change of V_{max} (cf above) the specific temperature effect could be derived. Using this approach the Q_{10} of the specific temperature effect was found to be 2.0 ± 0.3 (mean \pm SEM, n=8). This value agrees well with the temperature dependence of V_{max} during tetanic contraction of skeletal muscle (Clos Physiol Rev 1972 52 129).

Adrenergic stimulation of the heart increases the myocardial oxygen requirements due to increased mechanical activity and excessive myocardial consumption of free fatty acids (FFA). The purpose of the present study was to test whether ischemic injury of the myocardium might be aggravated by dopamine treatment. Comparison was made between the effects of dopamine (120 µg/min) and calcium (1.5 mEq/min) on myocardial ischemic injury following acute coronary artery occlusions in 12 anesthetized dogs. Ischemic injury was measured as the sum of S-T segment elevations in 9-11 epicardial ECG recordings (Σ ST).

Σ ST averaged 26 ± 4 mV at control occlusions. At reocclusions during inotropic stimulation Σ ST was 73 ± 12 mV with dopamine but only 41 ± 7 mV with calcium ($P < 0.005$) although the ventricular performance was similarly raised by the two drugs. Arterial concentrations of FFA rose by $258 \pm 61\%$ with dopamine but remained unchanged during calcium infusion.

Similar experiments were made after inhibition of lipolysis with β -pyridyl cartinol (9 mg/min). No differences in Σ ST or arterial FFA concentrations could be demonstrated during dopamine and calcium infusions.

It is concluded that stimulation of lipolysis contributes significantly to the enlargement of acute myocardial ischemic injury caused by dopamine.

The influence of anaphylaxis on ATP level and oxidative metabolism was examined in mast cells from sensitized rats. Mast cells were isolated (Fl 11) and resuspended in buffer (pH 7) including 1 mM Ca⁺⁺, 1 µg/ml human albumin and 1 mM pyruvate as substrate. After preincubation of the cells for 20 min at 37°C antigen (horse serum 0.7% final conc) or buffer (controls) was added and the incubation was continued up to 2 hr. ATP level or change in ATP was found with controls ($10.15 \text{ mol/mast cell}$). After anaphylaxis a significant decrease was obtained 25% after 1 min and 40% after 5 min with no further change up to 2 hr. Similar results were obtained when pyruvate was excluded. When 5 mM glucose was used as substrate no changes or differences were found between anaphylactic and control cells. CO₂ development in the control cells the CO₂ production from C14 pyruvate (1 mM) was found to increase linearly with time ($2.3 \times 10^{-16} \text{ mol CO}_2/\text{min/mast cell}$). After anaphylaxis a significant increase (approx. 30%) was found up to 2 hr. CO₂ production was completely inhibited by antimycin A (10^{-6} M) indicating that it represented oxidative metabolism. To examine whether an uncoupler of oxidative phosphorylation could mimic the effects of antigen the experiments were repeated with 10^{-4} and 10^{-5} M of 2,4 dinitrophenol instead of horse serum. Similar results were obtained. To conclude Anaphylaxis caused a decrease in the ATP level of the mast cells and an increase in their CO₂ production which could be the consequence of an uncoupling effect of oxidative phosphorylation induced by the antigen-antibody reaction. Grants: Danish Med Res Council and Danish Found. for the Advancement of Med Science.

Histamine release from mast cells induced by compound 48/80 is blocked by inhibitors of energy metabolism (Diamant and Uvnäs 1961) in contrast to when n-decylamine or chlorpromazine is used as releasing agent (Högborg and Uvnäs 1960 Prisk-Holmberg 1971)

After the addition of antimycin A ($0.2 \mu\text{M}$) to suspensions of mast cells the ATP content of the cells decreased to 1/3 of the original value in 2-3 min. Concomitantly the ability of the cells to release histamine when exposed to 48/80 decreased. The addition of glucose ($0.3-0.6 \text{ mM}$) to antimycin A treated cells partially restored ATP and concomitantly the ability to release histamine when exposed to 48/80 was restored.

To investigate if the energy dependence of 48/80-induced histamine release reflected an increased energy utilization during the release process the ATP content of mast cells was measured before and after histamine release had occurred. When cells with an intact energy production were used the content of ATP was unchanged after the addition of 48/80. However when cells were treated with antimycin A for 1 min the decrease of the ATP content was significantly greater after histamine release had been induced by 48/80 than in control samples or when histamine release had been induced by chlorpromazine or n-decylamine.

It is suggested that histamine release induced by 48/80 but not that induced by chlorpromazine or n-decylamine is accompanied by an increased utilization of endogenous ATP and that cells with an intact energy metabolism immediately restore the ATP level.

Johansen T and Chakravarty N (Dept of Pharmacology 200
Odense University 5000 Odense Denmark) THE RELATION
OF ADENOSINE TRIPHOSPHATE CONTENT OF RAT MAST CELLS TO
ANAPHYLACTIC AND COMPOUND 48/80-INDUCED HISTAMINE RELEASE

Pure populations of rat peritoneal mast cells were isolated by differential centrifugation in concentrated serum albumin. The cell suspensions in Krebs-Ringer solution were preincubated with varying concentrations of oligomycin to reduce the adenosine triphosphate (ATP) content of the mast cells. Histamine release was induced thereafter either by antigen (egg albumin) using mast cells from actively sensitized rats or by the polymeric amine, compound 48/80. ATP was assayed by the bioluminescence technique and histamine by the fluorometric method.

Oligomycin gave similar but not identical dose response curves for the reduction of ATP content and the inhibition of histamine release. In general the degree of reduction of the ATP content showed a good correlation with the inhibition of histamine release. Eighty to 100 per cent reduction of the ATP content was associated with almost complete inhibition of the release.

The observations are consistent with the view that energy requiring processes are involved in the anaphylactic histamine release from mast cells.

201 Anderson, P. S. A. Slorach and B. Uvnäs (Department of Pharmacology, Karolinska Institutet Stockholm 60 Sweden) CORRELATION BETWEEN HISTAMINE RELEASE AND ULTRASTRUCTURAL CHANGES IN NORMAL AND SENSITIZED RAT MAST CELLS IN VITRO EVIDENCE FOR AN EXTRACELLULAR RELEASE OF HISTAMINE

Mixed cell suspensions were taken from peritoneum and pleura in normal and sensitized (egg-albumin and pertussis vaccine) male Sprague-Dawley rats. The cells were incubated either with 0.4 µg/ml compound 48/80 at 17°C or 5 µg/ml egg albumin at 25°C (sensitized cells). Samples were taken for histamine assay and electron microscopy. The latter were fixed in 2% glutaraldehyde and 1% OsO₄, dehydrated and embedded in Vestopal W or Araldite. Lanthanum nitrate and hemoglobin were used in some experiments as extracellular tracers. Untreated mast cells or mast cells taken before the histamine release started at 10 sec or 30 sec (sensitized cells) showed the ultrastructural appearance of normal non-degranulating mast cells with homogeneous electron dense granules surrounded by perigranular membranes. The lanthanum and hemoglobin precipitates were adsorbed to the cell membrane but none was found inside. The initial changes were always found in the most peripherally located granules. Such granules showed a swollen appearance and a decreased electron density compared to normal granules. All changed granules were shown to be in communication with the extracellular space. Histamine release were correlated to the granule changes and increased to 65% after 3 min with compound 48/80 and 21% with antigen. During this time the above changes affected more and more mast cells and penetrated more deeply into the mast cells.

202 Sörenby, L. (AB Draco Research and Development Laboratory Lund Sweden): THE INHIBITORY EFFECT OF TERBUTALINE ON THE ANTIGEN-INDUCED RELEASE OF HISTAMINE FROM THE GUINEA-PIG LUNG.

Guinea-pigs were sensitized with ovalbumin in Freund's complete adjuvant. Two to four weeks later the lungs were challenged with ovalbumin in vitro.

The incubation fluids and boiled tissue extracts were assayed on the atropinized guinea-pig ileum. The amplitudes of the rapid contractions were compared with those of known histamine solutions and here referred to as histamine.

There was an increase of histamine in the incubation fluid and a concomitant decrease of tissue histamine after treatment with ovalbumin. 0.01-1.0 mg/ml Terbutaline $2 \cdot 10^{-8}$ - $2 \cdot 10^{-6}$ M added immediately before ovalbumin counteracted the histamine release. Propranolol $2 \cdot 10^{-6}$ M and INPEA $2 \cdot 10^{-5}$ M blocked the inhibitory effect of terbutaline. The effective terbutaline doses are in accordance with those for dilatation of the guinea-pig trachea. It is concluded that terbutaline counteracts the antigen-induced release of histamine from the guinea-pig lung. The effect is mediated by β -receptor stimulation.

Melander A P Sundler & U Westgren (Departments of Pharma- 203
cology and Histology, University of Lund Sweden); SIGNIFICANCE
OF THYROID MAST CELLS IN THE REGULATION OF THYROID ACTIVITY

The significance of thyroid mast cells in the regulation of
thyroid activity was studied in vivo and in vitro on rats mice
and calves. A combination of histochemical chemical and phys-
iological procedures was used.

Rodent thyroid mast cells were found to contain 5-hydroxy-
tryptamine and histamine whereas the calf thyroid mast cells
contained dopamine and histamine. The number of thyroid mast
cells could be correlated to the plasma TSH level and an in-
crease in the plasma TSH level stimulated the formation of
mast cells within but not outside the thyroid. TSH also evok-
ed a rapid release of amines from thyroid mast cells. Release
of mast cell contents induced by compound 48/80 was accompanied
by increased secretion and synthesis of thyroid hormone and
by an increased thyroid blood flow. These effects could be mim-
icked by the amines. TSH and compound 48/80 induced an increase
in thyroid blood flow only when a significant number of mast
cells was found in the thyroid.

It seems probable that the formation of thyroid mast cells
is controlled by TSH, and that the TSH-regulated activation of
the thyroid is facilitated and partially mediated by amines
that are released from thyroid mast cells by TSH.

Lie M, Johannessen J and Kill P (Institute for Experimental Medical 204
Research, University of Oslo, Ullevål Hospital, Oslo, Norway)
EFFECT OF CYANIDE ON CORTICAL AND OUTER MEDULLARY METABOLIC RATES

Cyanide blocks active tubular Na reabsorption but it is
controversial to what extent all segments of the tubules are affected
(M. Martinez-Maldonado et al. Am J Physiol 217:1363-1368 1969
S W Weinstein and R M Klose Am J Physiol 217: 498-504 1969).
To examine the distribution of the effects on renal metabolism after
cyanide injection into the renal artery, cortical and outer medullary
metabolic rates were measured in anaesthetized dogs using the heat
accumulation technique. OPR fell by $43.5 \pm 3.2\%$ and tubular Na
reabsorption by $53.3 \pm 3.5\%$ after cyanide infusion at rates of 10
 $\mu\text{mol/min}$. Metabolic rate was reduced in the outer medulla by
 $56.1 \pm 2.3\%$ in the cortex by 54.3 and 3.2% the difference not being
statistically significant. After distal blockade of sodium
reabsorption with ethacrynic acid and chlorothiazide, cyanide had no
further effect on outer medulla metabolic rate whilst cortical metabolic
rate was reduced by $37.9 \pm 3.4\%$.

It is concluded that cyanide blocks active sodium reabsorption to an
equal extent in proximal tubules and ascending limb of Henle's loop
in contrast to the effects of ethacrynic acid and chlorothiazide
mainly located in the distal nephron.

205 Møller, O. J. (Institute of Physiology University of Aarhus Denmark): DOES THE BRUSH BORDER OF THE PROXIMAL TUBULE CONTAIN A (Na + K)-ACTIVATED ATPase

Whether potassium is transported actively or passively in the proximal tubule of rabbit kidney is a matter of dispute. Because of the electrical and concentration gradients, active transport (if it occurs) must take place across the luminal membrane. The presence here of a (Na + K)-ATPase would strengthen the thesis of active K^+ -transport at this interface.

Brush border fragments of proximal tubule were prepared according to Thuneberg and Rostgaard (Exp. cell res. 51 (1968) 123). By electron microscopy they appeared to be almost uncontaminated by other membrane material. Such preparations had a (Na + K)-activity of 0.6 $\mu\text{moles Pi split/mg protein per min}$ and an alkaline phosphatase, a marker enzyme for brush border, activity of 1.8 $\mu\text{moles Pi split/mg protein per min}$.

The preparations were treated by gradient centrifugation as well as by continuous free-flow electrophoresis, but it did not prove possible to alter the ratio of (Na + K)-ATPase/alkaline phosphatase by more than two times. Although it is likely that (Na + K)-ATPase is present in brush borders, its presence only in contaminating (non brush border) membrane fragments could not be excluded.

206 Saltelli, M. and H. R. Ulfendahl (Institute of Physiology and Medical Biophysics University of Uppsala, Sweden): INTRACELLULAR CHLORIDE ACTIVITY OF THE PROXIMAL TUBULES IN THE RAT KIDNEY

Cl^- reabsorption in the proximal tubules is generally accepted to be but there still remains the problem whether the transport route is or intercellular. Several factors are of importance for transcellular Cl^- transport, among other the intracellular chloride activity. The aim of this investigation was to study the intracellular chloride activity with liquid ion exchange electrodes.

The electrode was of a glass open tip type with an outer diameter of less than 1 micron, where the very tip was filled with a chloride selective liquid. The reference electrode was an Ag/AgCl electrode with a KCl bridge and had the same capillary design. The in vivo measurements were made on rats where the left kidney was reached via a flank incision and immobilized in a cup. With micromanipulators the proximal tubular wall was punctured.

The potential measured is the sum of the chloride and the transmembrane potential. In another series the transmembrane potential had to be measured with Ling-Gerard electrodes. As the potentials were generally transient and showed a large amplitude scattering, the evaluation of intracellular chloride activities became very difficult. The results indicate an intracellular chloride activity of 10-50 mM.

Karlmark B, Persson A E G, Wistrand P, and E Ågerup (Dept of 207
Physiology and Med Biophysics and Dept of Pharmacology Univ of Uppsala
Sweden) ON THE QUESTION OF AN INTRALUMINAL CARBONIC ANHYDRASE
AS A FACTOR IN THE RENAL BICARBONATE REABSORPTION

The postulated mechanisms behind the bicarbonate reabsorption from the
proximal tubule of the kidney include the combination of filtered bicarbonate
ions and secreted hydrogen ions to form intraluminal carbonic acid. This
substance would rapidly decompose into water and carbon dioxide under the
influence of carbonic anhydrase which is located on the luminal side of the
tubular cell. As carbon dioxide probably is the major substance in this
chain which is reabsorbed, the specific inhibition of this postulated
enzyme ought to diminish the rate of bicarbonate disappearance from the
tubular lumen.

The experiments were performed on white rats with one kidney prepared for
micropuncture studies. Bicarbonate solutions were instilled into the proxi-
mal tubules using the Gertz technique. The rate of pH decrease (rate of
bicarbonate reabsorption) in the droplets was measured with an antimony
micro electrode system. Four different solutions were used: all of them
containing 150 mM NaHCO_3 : a) pure bicarbonate, b) bicarbonate + Acetazo-
lamide, c) bicarbonate + a dextran-coupled inhibitor and d) bicarbonate + a
new polar inhibitor. The carbonic anhydrase inhibitors in the two last
solutions were designed to act exclusively intraluminally.

The results show a markedly delayed acidification using the acetazolamide
solution as compared to the others. The rate of acidification using the
intraluminal inhibitors was not different from that of the unmixed bicarbo-
nate solution. - Thus, the results do not show any evidence for an intra-
luminal carbonic anhydrase activity.

SKJELVÅG, S.J. (Institute for Experimental Medicine, University of Copenhagen) 208
REMARKS: 1. PHYSIOLOGICAL IMPLICATIONS OF RENIN KINETICS

To establish the influence of substrate concentration and co-factors on
reaction velocity, renin substrate was purified from plasma of nephrecto-
mized sheep, rabbits and rats and also from pregnant women. Kinetic
constants for the semi-purified homologous reactants were compared with
those from unpurified plasma in 0.16M saline-phosphate buffer at pH 7.4.
All systems contained 0.001 M EDTA and were angiotensinase-free. Initial
velocity of angiotensin formation was determined by bio-assay in the
ganglion-blocked rat. Sheep substrate was purified 1,200 times over nor-
mal plasma (8 mU/mg protein) in 2 steps on ion-exchange and gel-filtration
chromatography with better than 80% recovery. At this purity, K_m for the
homologous sheep reactants ($2 \mu\text{M}$) was identical with that found in unpurified
whole plasma. The homologous rabbit ($K_m = 2 \mu\text{M}$) and rat ($K_m = 2.5 \mu\text{M}$) and
heterologous reactions (K_m greater than $3 \mu\text{M}$) could also not be established as dif-
ferent between purified and unpurified preparations.

Maximum velocity for the human reaction was not reached even at substrate
concentrations of $10 \mu\text{M}$ ($0.14 \mu\text{g/mg protein}$). K_m could not be accurately
determined but is predicted as considerably greater than $3 \mu\text{M}$. K_m for the
heterologous reaction human renin on sheep substrate was $0.29 \mu\text{M}$ and readily
determined. In all of these species, normal substrate concentration is at
or near the first order range and exerts control over the in vitro pro-
duction rate of angiotensin. No evidence for reaction co-factors was en-
countered. Physiological studies of plasma renin concentration and sub-
strate in rabbits and man indicated that first order kinetics also has an
important influence on in vivo renin activity.

RENAL HANDLING OF PHENOL RED A COMPARATIVE STUDY ON THE ACCUMULATION OF PHENOL RED AND p-AMINOHIPPURATE IN RABBIT KIDNEY TUBULES IN VITRO

The characteristics of renal accumulation of phenol red and p-aminohippurate (PAH) have been compared. It has been found that the aerobic accumulation of phenol red is about 4-5 times as high as that of PAH. Furthermore phenol red is accumulated by renal tissue under anaerobic conditions in contrast to PAH. The aerobic accumulation of phenol red as compared to PAH is less susceptible to inhibition by probenecid, 2,4-dinitrophenol (DNP) and octanoate whereas fumarate and succinate affect the aerobic accumulation of both phenol red and PAH to the same degree. Finally studies on renal homogenates showed that phenol red is bound to constituents of renal tissue in particular to the microsomal and mitochondrial fraction. It is concluded probenecid, DNP and octanoate cause more inhibition of organic anion transport than fumarate, succinate and PAH because of lipophilic interaction with the membrane.

210 Christensen S (Department of Pharmacology University of Copenhagen Denmark); RENAL CONCENTRATION ABILITY OF RATS WITH LITHIUM-INDUCED POLYURIA

Rats treated chronically with subtoxic doses of Li develop complete polyuria. It is uncertain to what extent the disturbance is due to primary polydipsia or to impaired renal concentration ability. To elucidate this problem, dehydration experiments were carried out in (1) rats with polydipsia induced by replacing drinking water with 5% glucose, (2) rats with pituitary diabetes insipidus and (3) Li-polyuric rats. Each group was deprived of water for 72 hours or until 20% loss of body weight. Weight loss, diuresis and urine osmolality were recorded every 6 hours and serum osmolality at the end of the deprivation period. The initial urine osmolalities were 100-300 mosm/kg in all groups. Maximum urine osmolalities during dehydration were (1) 2540-2880, (2) 970-1160 and (3) 960-1880 mosm/kg. Terminal serum osmolalities were (1) 310 ± 1 , (2) 246 ± 9 and (3) 382 ± 34 (S.D.) mosm/kg. In the Li-group (3) the maximum urine osmolalities were linearly correlated with the initial values. It is concluded that lithium-polyuria in rats is due to decreased renal concentration ability rather than to a polydipsic action of Li.

Zebra Finches (ZF) from Western Australia were previously observed to drink saline up to 0.8 M whereas domesticated ZF only drank 0.3 M NaCl and excreted droppings of maximally 700 mOsm. ZF caught at Mileura 800 km NE of Perth (annual rainfall 5 cm) were studied. Drinking of 0.8 M NaCl was confirmed but the average osmolality of the urine (supernatant of the lower end of the droppings after spinning at 26000 g) was only 1027 mOsm. The osmotic urine to plasma ratio was 2.79 identical to that in the dehydrated state (2.78). This indicates that ZF of arid zones most likely represent a genetically select subgroup.

In order to investigate why these ZF can drink fluids more concentrated than the droppings the water turnover was measured in the dehydrated state. The food intake was recorded and the droppings were collected quantitatively under oil. The 24 hr water turnover (preformed and metabolic water) was estimated to 24% of the total body water. This calculation was confirmed by measurement of the 24 hr turnover of HTO (23%). Drinking of 0.8 M NaCl seemed to occur since the intake only amounted to 10% of the water turnover in the dehydrated state. It increased the average urine Na and Cl concentrations from 7.6 and 39.8 meq/l respectively in the dehydrated state to 117 and 161 meq/l. This probably caused a greater water absorption in the cloaca.

In a number of smooth and striated muscle preparations La^{3+} can displace Ca^{2+} from surface (stabilizing) sites and inhibit the uptake of Ca^{2+} to less superficial sites or stores. In some systems (especially guinea pig ileal longitudinal smooth muscle) La^{3+} may also alter the mobility of Ca^{2+} present at less superficial sites; this effect is an indirect one and exists as a function of the degree of interaction between superficially-bound Ca^{2+} and Ca^{2+} located at less superficial sites. Conversely the increased Ca^{2+} uptake elicited with either caffeine (in frog sartorius muscle) or nicotine (in frog rectus abdominis muscle) is not blocked by La^{3+} . Thus in each type of muscle employed it is necessary to exclude the possibility that alterations in ^{45}Ca content may result in part from La^{3+} -resistant effects on uptake or efflux of ^{45}Ca . Exposure to La^{3+} as well as depletion of Ca^{2+} inhibited K^{+} -induced contractions more than those elicited with norepinephrine or histamine in rabbit aortic strips with acetylcholine in rat uterus and with caffeine in frog sartorius muscle. However La^{3+} inhibited only the initial portion of the response of frog rectus abdominis muscle to high K^{+} , nicotine and acetylcholine whereas Ca^{2+} -depletion completely blocked responses to all three of these agents.

All of the specific effects observed with La^{3+} can be directly related to an interference with the manner in which cellular Ca^{2+} is taken up, stored and utilized in each distinct type of muscle. Use of La^{3+} in this manner can help identify mechanisms by which various pharmacological stimuli utilize Ca^{2+} in different types of smooth and striated muscle.

213 Batra, S (Dept of Pharmacology University of Lund Sweden);
EFFECTS OF CERTAIN DRUGS ON Ca UPTAKE AND Ca RELEASE BY MITOCHONDRIA
AND SARCOPLASMIC RETICULUM OF FROG SKELETAL MUSCLE

The effects of quinidine chlorpromazine and caffeine on Ca uptake and Ca release by mitochondria and fragmented sarcoplasmic reticulum (FSR) were studied. Quinidine (1-2 mM) released considerable Ca from preloaded mitochondria but had little effect on preloaded FSR. The uptake of Ca both by mitochondria and FSR was inhibited by higher concentrations (2 or 1 mM) of quinidine but the inhibition of mitochondrial Ca uptake was much greater. With lower concentration (0.4 mM) there was no significant effect on Ca uptake by FSR but a 48% inhibition of mitochondrial Ca uptake was observed.

Chlorpromazine (0.01-0.1 mM) inhibited Ca uptake by both mitochondria and FSR but the inhibition in the case of FSR was weaker than mitochondria. Only the highest concentration (0.1 mM) of chlorpromazine caused a release of Ca from mitochondria or FSR. Caffeine (2-10 mM) inhibited Ca uptake both by mitochondria and FSR and again the inhibition of Ca uptake by mitochondria was greater than of FSR. Caffeine (10 mM) in contrast to quinidine released Ca from FSR and not from mitochondria.

Ca releasing concentrations of these drugs were comparable to those reported to elicit contractions of living muscle. Lower concentrations which inhibited Ca uptake were comparable to those which potentiate twitch.

Haffner J F W B -I Iesheim & J Setekleiv(Institute of Pharmacology University of Oslo Norway);K-EFFLUX AND THE ADRENERGIC RESPONSE IN ISOLATED RABBIT STOMACH MUSCLE

- studies presented (Haffner J F W B & I Iesheim & J Setekleiv Acta pharmacol et toxicol 1972 31 412; 1973 1974 in press).
were carried out to determine whether the variable mechanical responses to adrenergic agents in rabbit stomach muscle were correlated with alterations in K-efflux. Fundus and antrum strips were loaded with ^{86}K mounted in a perfusion chamber and washed with Krebs-solution. Adrenaline noradrenaline phenylephrine or isoprenaline was added after an equilibration period of 28-32 min. Tension and K-efflux were recorded. Phenylephrine noradrenaline and adrenaline produced significant ($p < 0.05$) increases in K-efflux both when the mechanical response was excitatory (increased tone in the fundus initiation of contractions in the antrum strips) and inhibitory (inhibition of contractions in antrum strips) and also when no mechanical response occurred. Nor was any correlation found between the magnitude or time course of the mechanical response and the increase in K-efflux from fundus strips. Isoprenaline had no effect on K-efflux.

It is concluded that the increase in K-efflux produced by alpha-receptor stimulation appear to be of no causal importance for the mechanical response in rabbit stomach muscle.

Nasheim B I (Institute of Pharmacology University of Oslo 215 Norway) ACTION OF NORADRENALINE AND ISOPRENALINE IN THE OESTROGEN AND THE PROGESTERONE DOMINATED RABBIT UTERUS

Hypogastric nerve stimulation contracts uteri from oestrogen-dominated rabbits and relaxes uteri from progesterone dominated rabbits. It has been postulated that the adrenoreceptors in the oestrogen dominated myometrium are predominantly excitatory (α -receptor) and in the progesterone dominated myometrium predominantly inhibitory (β -receptor). This hypothesis has been examined by in vitro stimulation with noradrenaline and isoprenaline of muscle strips taken longitudinally from the uterine horns. The strips showed spontaneous activity and isoprenaline caused an inhibition of this activity. There were no differences in the dose response curves from oestrogen and progesterone dominated uteri.

Noradrenaline increased the frequency and the basal tone of the contractions to the same degree in oestrogen and progesterone dominated strips. In oestrogen dominated strips the force of the contractions was increased up to three times the force of the spontaneous contractions. In progesterone dominated strips the force of the contractions was not increased.

It is concluded that the difference between the oestrogen and progesterone dominated uteri seen on sympathetic nerve stimulation depends on different reactions to α -receptor stimulation.

Lagercrantz, H. (Dept. of Physiology I Karolinska Institute Stockholm 216 Sweden): NORADRENALINE AND PROTEIN RELEASE FROM ISOLATED SYMPATHETIC NERVE VESICLES

Noradrenaline (NA) has been assumed to be released together with its storage components from sympathetic nerve vesicles by exocytosis in the same way as catecholamines (CA) are secreted from chromaffin granules (see Smith & Winkler Handbook of Experimental Pharmacology XXXIII:537 1972).

In vitro studies of highly purified nerve vesicles isolated from bovine splenic trunk have demonstrated significant differences between these vesicles and those isolated from the adrenal medulla: 1) While the chromaffin granule consists of about 80 % of a water soluble CA: ATP: protein complex, the nerve vesicle preparation was found to contain less than 20 % soluble proteins and dopamine β hydroxylase. Only trace amounts of chromogranin A and also less ATP (NA/ATP ratio = 9/4). 2) Incubation at 30°C resulted in a rapid spontaneous release of NA (48 % after 10 min) mainly independent of protein and ATP release (only 2 % dopamine β hydroxylase was released after 10 min). The chromaffin granules release CA much slower under similar conditions and there is a parallel loss of soluble proteins and ATP (see Stjärne Handbook of Experimental Pharmacology XXXIII:231 1972).

Conclusion: The nerve vesicle seems to store only one fifth of its NA in a water soluble complex with proteins and ATP. Such a complex is assumed to be released by exocytosis.

217 Serck-Hanssen Guldberg (Institute of Pharmacology, University of Oslo, Norway): SECRETION OF ADRENOMEDULLARY CATECHOLAMINES EFFECTS OF THEOPHYLLINE AND PROPRANOLOL

Methyl xanthines have been shown to stimulate the secretion of catecholamines from the adrenal medulla (Berkowitz B A and Spector, S Eur, J Pharmacol 1971 13 193) and to potentiate the secretion of catecholamines Induced by cAMP (Peach, M J, Proc Nat Acad Sci 1972 69 834)

In the present work the secretion of catecholamines from retrogradely perfused bovine adrenal has been studied. Acetylcholine in half maximum dose 10^{-6} M caused the release of adrenaline and noradrenaline in ratios of approximately 2/3. The presence of 1 mM theophylline in the perfusion medium (Tyrode's buffer) potentiated the effect of acetylcholine about 30 % and produced a relative increase in the amount of adrenaline released. The effect of theophylline was also observed in glands blocked with 10^{-6} M atropine. Propranolol in a concentration of 2×10^{-6} M at which it had no membrane stabilizing effect, reduced the acetylcholine-induced secretion about 20 %.

The present results indicate that the acetylcholine-induced secretion of catecholamines from the adrenal medulla is partly mediated by a β -adrenergic receptor. As the β -receptor is closely associated with the adenyl cyclase the secretion from the medulla seems partly to be controlled by cAMP.

Wang, E (Institute of Pharmacology University of Oslo): STIMULATION OF THYMIDINE UPTAKE BY ACTINOMYCIN D IN HEPATOMA CELL CULTURE

Preincubation with actinomycin D for 2 hours increases the uptake of 3 H-thymidine into the acid-soluble fraction of MH₁C₁ cell cultures with a linear log dose-response curve from 0.01 μ g/ml to 10.0 μ g/ml. Actinomycin D at a dose of 1.0 μ g/ml gave 200-250 % stimulation; increase in the dose above that which completely blocked RNA synthesis still increased thymidine uptake. A parallel increase in activity in the acid-insoluble fraction was found. The effect was detectable after 5 minutes but was maximal first after 1-2 hours treatment with the drug. This effect could not be blocked by cycloheximide. Actinomycin D preincubation for 2 hours inhibited 3 H-hypoxanthine uptake into the acid-soluble fraction with a linear log dose-response curve from 0.01 μ g/ml to 10.0 μ g/ml; 50 % inhibition was seen at 0.75 μ g/ml. 10.0 μ g/ml gave 73 % inhibition. Actinomycin D preincubation altered the V_{max} of the thymidine and the hypoxanthine uptake but did not change the K_m s of the two reactions. Actinomycin D did not change the activity of thymidine kinase in vitro.

Garberg, L Borg K-O Olbe L Sjöstrand ■ E Sundell G 215
(Pharmacological dept AB HM ele Malmödal Sweden and Surgical
Clinic II Sahlgrenska Hospital Gothenburg Sweden): EVALUA-
TION IN THE DOG OF A NEW GASTRIC ACID SECRETION INHIBITOR
H 81/75 a potent inhibitor of gastric acid secretion in the
rat (Sundell et al) has been studied in several experimental
models in the dog

In doses of 20-50 mg/kg i v H 81/75 was found to inhibit
gastric acid secretion in dogs in which vagal activity has
been stimulated. However when pentagastrin or histamine were
used as stimulators no effect was found. General pharmacologi-
cal studies have shown that the compound is not to be con-
sidered as a general anticholinergic agent.

Pharmacokinetic studies using ³H-labelled compound have been
carried out in the dog. A difference in the rate of elimination
of H 81/75 in the dog and the rat was found.

It is concluded that H 81/75 is a more limited inhibitor of
gastric acid secretion in the dog than it is in the rat. Data
support a peripheral site of action in the dog.

Sundell G Borg K-O Garberg L Palmer L Sjöstrand 220
S E (Pharmacological dept AB H Mäele Malmödal Sweden) EVA-
LUATION OF A NEW GASTRIC ACID SECRETION INHIBITOR H 81/75 IN
THE RAT

H 81/75 is a carbamate structure: N-(2-methoxyethyl)-propyl-
carbamate. Its effect on gastric acid secretion in the rat has
been studied in different experimental models including the
pylorus ligated rat (Shay rat) and the acute fistula rat both
under basal conditions and on pentagastrin histamine or A-DG
stimulated secretion.

In the Shay rat ED₅₀ for inhibition of gastric acid secretion
was 1.5 mg/kg.

In the acute fistula rat duration studies were performed.
H 81/75 showed more than 40 % inhibition of basal gastric acid
secretion during 4 hours after administration of a single dose
6 mg/kg. A dose-dependent inhibition of stimulated gastric acid
secretion was found.

Pharmacokinetic studies have been performed with ³H-labelled
compound in the acute fistula rat. This showed a linear rela-
tionship between plasma concentration and antisecretory effect
on basal gastric acid secretion.

Thus H 81/75 was found to be a potent inhibitor of both sponta-
neous and stimulated gastric acid secretion in the rat.

221 Thiringer G & Svedmyr Nils (Lung Clinic University of Göteborg Sweden) COMPARISON OF I V ADMINISTERED AND INHALED TERBUTALINE WITH DOSE EFFECT CURVES IN PATIENTS WITH CHRONIC OBSTRUCTIVE LUNG DISEASE

Tremor is the most usual side effect when giving the new relatively selective adrenergic β_2 stimulators. Tachycardia is the dose limiting effect only in few patients.

We have recorded dose response curves for the effect of terbutaline given i v and by inhalation on the bronchi ($FEV_{1.0}$), heart rate, blood pressure and muscle tremor. Terbutaline given i v to patients with chronic obstructive lung disease in doses that did not relax the bronchial muscle maximally more than doubled the tremor and increased the pulse rate about twenty beats/minute. The tachycardia is mainly due to peripheral vasodilatation and reflexogenic heart stimulation. When terbutaline was given by inhalation the same bronchial relaxation was obtained without any effect on circulation or muscle tremor indicating a local effect. The acute margin of safety was notable as the pulse increase after 63 inhalations of this long acting substance was only about twenty beats/minute. Conclusion: It was not possible to obtain maximal bronchodilatation with terbutaline given i v in patients with chronic obstructive lung disease without side effects. When terbutaline was given by inhalation much better relaxation of the bronchial muscle could be reached before the side effects occurred.

Palkonen O., 1941 E, Larmi T. K. I & Kärki N. T. (Departments Pharmacology and Surgery University of Oulu, Oulu, Finland):

LIVER DRUG-METABOLIZING ENZYMES IN DIFFERENT DISEASES

Clinical and experimental studies have demonstrated impairment of oxidative drug metabolism in diseases affecting liver (for ref. see Aurenhan A. Acta Chirurgica Scandinavica 1972 Suppl. 424). Earlier we have studied in vitro activities of drug-metabolizing enzymes in liver biopsy samples from patients with cholelithiasis (CL) (Palkonen O. et al. Scand J Clin Lab Invest 1973 31 suppl 130-24).

In this study liver samples were obtained from patients with obstructive icterus (8), cirrhosis (7) and cancer (13). Samples were studied with respect to cytochrome P-450 and b5 NADPH-cytochrome c reductase, 3,4-benzpyrene and aniline hydroxylases, aminopyrine N-demethylase and hexobarbital oxidase. Values obtained were compared with those of CL-patients (36).

Mean levels of drug-metabolizing enzymes were decreased about 10 - 40 % in obstructive icterus and 20 - 70 % in cancer patients (mostly stomach carcinomas with or without metastases). The impairment of drug metabolism increased when the histological appearance of the liver sample changed from normal to malignant. No significant decrease in enzymes was observed in patients with hepatic cirrhosis. Also no significant difference was detected between CL-livers with normal histological appearance and CL-livers with mild to moderate inflammatory and degenerative changes.

The effects of thioridazine and thiothixene were studied by a double blind technique on 40 schizophrenic patients. The doses were adjusted for optimal clinical effects and ratings for therapeutic and side-effects made after three and eight weeks of treatment. No statistically significant differences were obtained between the two drugs or between any of the two drugs and the previous medication.

Plasma levels were estimated by a fluorometric technique previously described by us after three and eight weeks of treatment. No correlation was obtained between plasma levels and clinical effects for either thioridazine or thiothixene. Between doses and plasma levels a clear correlation was found for both drugs after three weeks of treatment. After eight weeks this correlation remained for thioridazine but not for thiothixene. By that time serum levels of thiothixene had decreased about three times indicating strong enzyme induction.

Generic equivalence was observed in an earlier oral absorption study of three diazepam preparations (Kangas et al. Annals Clin Research 1973 to be published). Diazepam (D) (Tensopam) 10 mg was given orally to 12 chronic alcoholics (I) most of whom were heavy drinkers at the beginning of the alcohol free period and to 14 controls (II). D was determined gas chromatographically with a 63Ni EC detector. During the absorption phase of D at 2 and 3.5 h following administration of the tablet the blood levels of D were reduced by 43 % ($p < 0.01$) and 32 % ($p < 0.001$) respectively in I compared with II (140 ± 13 and 88 ± 12 ng/ml resp.). Seven subjects in I showed very low values of D during the absorption phase (< 45 ng/ml). Neuroleptic drugs (chlorprothixene, perphenazine or chlorpromazine) were given to 9 of the subjects in I but the earlier study has shown that this treatment does not alter the absorption profile of D compared with that seen in controls. Two of the 5 alcoholics who had taken dichloralphenazone the previous evening showed a similar rise in the absorption of D as that found in II. It therefore seems unlikely that these drugs inhibit the rise of D in the absorption test in alcoholics. The reduced values found in I may be due to disturbances in the gastro-intestinal absorption of D or to processes involving the portal vein or liver. Determination of D at 6, 12, 24, 36 and 48 h showed that its elimination as reflected by its plasma half life was slightly slower in I than in II.

225 Kanto J Iisalo E Lehtinen V Salminen J (Department of Pharmacology Turku University and Clinic of Psychiatry Turku University Central Hospital Turku Finland) CORRELATION BETWEEN PLASMA CONCENTRATIONS AND PSYCHIC EFFECTS OF DIAZEPAM IN LONGTERM TREATMENT

Plasma concentrations of diazepam (d) and N-demethyldiazepam (Nd) has been followed gaschromatographically for 5-24 weeks in 8 neurotic out patients receiving d 15 mg daily. After the maximum concentration in 1-6 weeks (d 179-642 ng/ml and Nd 212-1313 ng/ml) both d and Nd had a significant tendency to decrease (d 62-396 ng/ml and Nd 180-594 ng/ml) possibly due to a faster hepatic microsomal metabolism of d.

The psychic condition was tested with Taylor's Manifest Anxiety Scale at intervals of one month and the condition was assessed both by the psychiatrist and a patient himself at intervals of one week. The stepwise improvement in neurotic symptoms achieved by the therapy during the first month remained unaltered during the six following months. After 4-5 weeks therapy no correlation was detected between d and Nd plasma concentration and psychic condition.

The steady state concentrations of d and Nd in some other patients using d for 2-5 years were about 1/5-1/10 of those achieved by the same dose in an acute experiment. In some of those patients the dose of d had partially lost its effectiveness after 0.5-2 years of therapy and the dose had to be increased.

Hanhijärvi M Penttilä I Pekkarinen A & Hakulinen A (Department of Pharmacology University of Turku Finland) THE EFFECT OF ARTIFICIALLY FLUORIDATED DRINKING WATER ON THE PLASMA IONIZED FLUORIDE CONTENT IN CERTAIN DISEASES II

Plasma ionized fluoride concentration (IPF) was measured with the electrometric method in patients from areas with artificially fluoridated water (I) (1 ppm fluoride) and normal drinking water (II). A small but significant increase of IPF with advancing age was found in both areas. IPF was higher in renal insufficiency (Acta Pharmacol Toxicol 31:104 Suppl 1 1972) than in corresponding control.

In cor pulmonale IPF values in area I $2.7 \pm 0.4 \mu\text{M}$ (5) (SEM no. of patients) in the whole group of heart insufficiency $1.8 \pm 0.1 \mu\text{M}$ (50) and in severe heart insufficiency $2.2 \pm 0.3 \mu\text{M}$ (8) were significantly higher than in the corresponding control group of the same age $1.5 \pm 0.4 \mu\text{M}$ (178).

In a 6 years old boy with diabetes insipidus IPF in area I was $4.0 \mu\text{M}$ 4 times higher than in the control group of the same age. In cirrhosis with ascites IPF was $2.0 \pm 0.35 \mu\text{M}$ (3).

During pregnancy there was a significant decrease in IPF to $0.90 \pm 0.04 \mu\text{M}$ (40) probably indicating an accumulation of fluoride in fetal or maternal bone. When oedema complicated the pregnancy IPF was higher. A slight but significant decrease to $1.1 \pm 0.051 \mu\text{M}$ (49) in hypertensive patients was probably due to treatment. The conclusion is made that IPF is increased in diseases with marked generalized oedema. This is in agreement with a previous study by us showing a marked elevation in renal insufficiency.

In 159 control students the mean HR at rest 70.4 ± 0.6 /min increased to 104.0 ± 1.2 /min (33.6 ± 1.1 /min) ($p < 0.001$) during EP more in females (108.9 ± 1.8 /min $N=61$) than in males (100.9 ± 1.5 /min $N=98$) ($p < 0.001$). The increase of HR was higher at the time of the first period of EP (11.8 min) and the first question ($+37.7 \pm 1.1$ /min) than later ($p < 0.01$). 5 mg D perorally at least 1/2 hr before the EP reduced only very slightly HR during EP to 96.9 ± 1.6 /min ($p < 0.001$) and the rise of HR to $+27.0 \pm 1.4$ /min resp ($p < 0.001$) in 103 students as well as in both sexes in 35 females (to 102.0 ± 2.6 /min) ($p < 0.05$) and in 59 males (to 94.3 ± 1.9 /min) ($p < 0.01$). D (Valium) slightly reduced the rise of HR in the groups of moderate physical exercise ($p < 0.001$) from 109.1 ± 1.9 /min ($N=45$) to 93.3 ± 3.2 /min ($N=27$) ($p < 0.001$) or in physically inactive students from 107.2 ± 3.7 /min ($N=46$) to 101.6 ± 2.3 /min ($N=33$) with D ($p < 0.05$) while D did not reduce further already low HR during EP 95.5 ± 2.2 /min ($N=43$) in the group of athletes students with active physical training or runners to 93.1 ± 2.7 /min ($N=33$) nor the rise of HR from $+29.1 \pm 1.2$ /min to $+27.8 \pm 2.3$ /min. The rise of HR is higher during EP in the group of higher examination scores than in the group of lower examination scores ($p < 0.05$). D reduced HR ($p < 0.01$ and < 0.001 resp) and the rise of HR ($p < 0.01$ and < 0.001 resp) in these two groups of students as well as in the students who had failed ($p < 0.05$ $p < 0.05$) or students who had passed their examination ($p < 0.01$ $p < 0.01$).

Glycine-N acyltransferase (EC 2.3.1.13) catalyzes the glycine conjugation by condensing acyl coenzyme A with glycine to form the corresponding acylglycine derivative. In order to study the substrate (salicylate) induced activation of the conjugation system in detail (Irjala, K. Ann Acad Sci Fenn 1972 A V 154) fast and efficient purification procedure has been developed.

Rat liver mitochondria were suspended in glycine buffer (pH 9.6 ionic strength 0.1) containing 1% rivanol. They were sonicated to liberate glycine-N acyltransferase from the particles. All the mitochondrial protein powder can be used as starting material and the enzyme can be extracted with the aid of the same buffer. After sedimenting the insolubilized protein and rivanol-potassium complexes at 27000 g the supernatant containing glycine-N acyltransferase was placed in a CM-Sephadex H 50 column equilibrated with glycine buffer (pH 9.5 ionic strength 0.1). A convex NaCl gradient (from 0 to 0.2 mol/l) was used in elution. The glycine-N acyltransferase was eluted as a single peak with the main bulk of protein. The specific activity increased to 0.1 mol benzoyl glycine formed/min/g protein which indicates 200 fold purification compared with a crude mitochondrial extract. The enzyme preparation was very stable (no change in activity in four months at -20°C).

The present method is simple and reproducible and yields preparations in six hours pure enough for kinetic studies.

229 Rane And re (Depts of Pharmacol and Clin Pharmacol Karolinska Institutet Stockholm Sweden): N-OXIDE FORMATION FROM A TERTIARY AMINE IN HUMAN FETAL LIVER MICROSOMES

Human fetal liver microsomes contain an NADPH-dependent mixed-function oxidase system which catalyzes drug oxidations including C-oxygenations and N-dealkylations. The present study was undertaken to see if human fetal liver microsomes could catalyze the N-oxygenation of a tertiary amine N-N-dimethylaniline (DMA). Human fetuses were obtained via hysterotomy for the interruption of pregnancy for sociomedical reasons. Gestational ages varied between 13 and 21 weeks. The liver microsomes were found to N-oxidize DMA at rates varying between 0.3 and 2.4 nmoles DMA N-oxide formed/mg protein x min⁻¹. The reaction was dependent on NADPH. CO did not inhibit the reaction. Rather an increased formation of DMA N-oxide (about 17 %) was observed when CO was present in the medium. The findings are in agreement with results from N-oxygenation studies with rat and pig liver microsomes (of Ziegler & Mitchell Arch Biochem Biophys 1972 150:116). These studies indicated that N-oxygenation of tertiary (and secondary) amines is independent of the cytochrome P-450 enzyme system.

Various amines are known to exert toxic effects via their N-oxygenated metabolites. Therefore N-oxygenation of amines in human fetal liver is of interest from a teratologic point of view.

Supported by grants from Expressen's Prenatal Research Fund, the Association of the Swedish Pharmaceutical Industry, and the Swedish Medical Research Council (14 X-3902).

230 Marniemi, J. and Hänninen, O. (Department of Physiology University of Turku Finland): KINETIC PROPERTIES OF LIVER UDP GLUCURONYLTRANSFERASE WITH ¹⁴C LABELED p-NITROPHENOL AS SUBSTRATE

The kinetic studies of UDP glucuronyltransferase have been hampered by the lack of a purified enzyme preparation free from contaminants and by the lack of a sensitive enzyme assay. p-Nitrophenol has however been used recently often as the aglycone substrate of the enzyme because of its easy and rapid spectrophotometric determination. Unfortunately the method is not very accurate for the kinetic studies used.

We have studied the kinetics of UDP glucuronyltransferase using ¹⁴C labeled p-nitrophenol as substrate which increases the sensitivity about 100 times compared with the spectrophotometric method. The unconjugated aglycone and its glucuronide were separated from each other by the extraction with diethyl ether.

Fresh and frozen and thawed microsomes of rat and guinea pig appeared to have a transition point in their Lineweaver-Burk plots when the UDP glucuronic acid concentration was varied leading to two K_m-values for UDP-glucuronic acid. Transition as observed with frozen microsomes of beef liver. The reaction product uridine diphosphate inhibited UDP glucuronyltransferase of frozen beef liver microsomes competitively with UDP-glucuronic acid. The results of this study are in accordance with the findings of Winnes (BBA 26 394 1972 BBA 283 88 1972) obtained with the spectrophotometric method.

Rietanen E and O Hänninen (Department of Physiology University of Turku, Finland): EFFECT OF PROLONGED SALICYLIC ACID ADMINISTRATION ON THE MUCOSAL GLUCURONIDE SYNTHESIS IN THE RAT DUODENUM AND STOMACH 231

In previous experiments massive intragastric salicylic acid administration three times at 12 hr intervals resulted in gastric hemorrhage together with decreased UDP glucuronyltransferase activity in the gastric mucosa (Aitio A. et al: Mucosal drug metabolism and drug-induced ulcer Int. Congress on Exp. Ulcer Cologne 1972). The purpose of the present study was to follow changes in gastroduodenal glucuronide synthesis as related to the disappearance of gastric erosions in long-term salicylic acid treatment.

After a single dose of one mmole of salicylic acid per kg of body weight the mucosal erosions appeared in 12 hr and the UDP glucuronyltransferase activity decreased in the stomach. After three daily doses the erosions disappeared in most rats and the mucosal UDP glucuronyltransferase activity increased 2-fold in the duodenum and 1.5-fold in the stomach. The increase was however transient, and the enzyme activity returned to the control level within two weeks. The hepatic UDP glucuronyltransferase activity was also followed but the salicylic acid administration did not change it significantly. The gastric and duodenal protein content decreased after a single salicylic acid administration but turned back to normal in three days despite of continuation of drug administration.

The data show that during prolonged salicylic acid treatment the gastric erosions disappear and the mucosal glucuronidation capacity regenerates in the rat.

Grant: U.S. Public Health Service (AM-06018-11)

Ravn Jønsen A (Department of Pharmacology University of Copenhagen Denmark): THE METABOLISM OF A N METHYLATED BARBITURATE (ENIBOMAL- ^{14}C) IN THE RAT 232

In a recent investigation the distribution and excretion of unlabelled enibomal was reported (Ravn Jønsen Acta pharmacol. et toxicol. 1973 32: 401-407). Three metabolites were identified all derivatives of 5-isopropyl barbituric acid (B): 1-methyl-8-acetyl-B (II), 1-methyl-5-(2-oxo-3-hydroxypropyl)-B (VI) and 8-acetyl-B (VIII). Only 20 per cent could be recovered chemically in 72 hours.

In the present investigation ^{14}C enibomal (ring labelled) was synthesized from ^{14}C urea. The labelled barbiturate was administered i.v. (45 mg/kg specific activity 0.2 mCi/g). In a fractionated sampling period of 120 hours a total of more than 80% radioactivity was recovered with 70% in the urine, 10% in faeces while only 0.3% was found in the expiratory air. In the carcass was recovered 2%. By TLC the same metabolites was demonstrated as mentioned above and further 1-methyl-5-carboxymethylene-B (III) was identified. Besides these ether extractable metabolites also the remnant contained unidentified radioactivity but treatment with urease did not liberate $^{14}\text{CO}_2$. Of the major metabolites VI dominated in the beginning VIII in the later part of the sampling period. All metabolites were demonstrated both in urine and faeces.

233 Aitio A and Vainio H (Department of Physiology University of Turku Finland): INDUCTION OF MICROSOMAL XENOBIOTIC BIOTRANSFORMATION BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ADMINISTERED BOTH SINGLY AND IN COMBINATION

The mechanism by which phenobarbital (PB) stimulates the microsomal drug metabolism has been considered to be different from the mechanism by which polycyclic carcinogens produce their induction. In the present study we have examined the effect of phenobarbital (80mg/kg i p) and 3-methylcholanthrene (MC) (10 mg/kg in olive oil i p) when administered both singly and in combination on the hepatic microsomal drug oxidation (O-demethylation of p-nitroanisole, hydroxylation of 3,4-benzpyrene) and glucuronidation (p-nitrophenol, 4-methylumbelliferone) of the rat. Both MC and PB increased the O-demethylation of p-nitroanisole (6-fold and 9-fold respectively) whereas only MC increased the benzpyrene hydroxylase activity (13 fold). In combination their effect was additive on p-nitroanisole demethylase activity where PB decreased the extent of MC induction of benzpyrene hydroxylase. Both PB and MC enhanced the UDP glucuronyl transferase activity but no additive effect was obtained when the drugs were administered in a combination (calculated on microsomal protein basis). If calculated on total body basis an additive effect was observed on UDP glucuronyltransferase activity.

The two inducers used MC and PB thus seem to have either additive inhibitory or no effect on each other's actions depending on the enzyme studied.

234 Arvela, P (Department of Pharmacology, University of Oulu, Finland): THE IN VITRO EFFECTS OF LANTHANONS ON LIPID PEROXIDATION AND DRUG METABOLISM IN RAT

Lanthanons cause a severe liver injury which impairs the drug metabolizing capacity of rat liver (Arvela, P and Kerkki, T, *Experientia* 1971 27: 1189). The known hepatotoxicity of carbon tetrachloride (CCl_4) is supposed to be due to the enhanced lipoperoxidation in the liver (Recknagel, R O and Choshal, A K, *Lab Invest* 1966 15: 132). In order to find out if lanthanons act like CCl_4 , their effects in vitro on lipid peroxidation and drug metabolism were studied.

The lipid peroxidation was measured as malonaldehyde liberated and for test-reactions of drug metabolizing activity the hydroxylation of 3,4-benzpyrene, the glucuronidation of p-nitrophenol, the demethylation of aminopyrine and the amount of cytochrome P-450 were chosen. The lanthanons studied were lanthanum, cerium and erbium.

Contrary to CCl_4 , all lanthanons studied inhibited the lipoperoxidation when incubated in vitro with liver preparations and none of the studied drug metabolizing enzymes were significantly inhibited by lanthanon concentrations up to $10^{-4} M$.

It is concluded that the toxic effects of the lanthanons are different in vitro than those of CCl_4 , but more studies must be done to extend this assumption to in vivo circumstances.

Grant: The Finnish Culture-Foundation, Finland

Jensen, J. Aa (Department of Pharmacology University of Copenhagen Juliane 235
Marlervej 20 DK 2100 Copenhagen Denmark): THE RATE OF DISSOCIATION OF SALAZO-
SULFAPYRIDINE FROM ALBUMIN BINDING

The rate of dissociation of drugs from plasma protein binding is generally assumed to be of such an order of magnitude that it does not limit the rate of renal tubular secretion. However very little experimental data are available to prove or disprove this assumption.

The aim of the present investigation has been to determine the association (k_1) and dissociation (k_{-1}) rate constants of a drug-albumin binding. Salazo-sulfapyridine (S) was chosen because its binding to human serum albumin (HSA) gives rise to a reduction in optical density at 320 nm. The course of association of S to HSA was determined at 37° using a stopped-flow spectrophotometer (Durum). HSA from Behringwerke (20 µM) in a Tyrode solution (pH 7.4) was mixed with equal volumes of 2 µM - 40 µM S (donated by Pharmacia).

For three types of binding sites (Jansen, Acta Pharmacol Toxicol 1972 31 Suppl I 88) k_1 and k_{-1} were determined by simulating the course of association on a digital computer using steps corresponding to 1 µsecond. The three association constants and the number of sites of each type.

At physiological and therapeutic conditions only binding to the sites with highest affinity are of importance. The half-time of dissociation from these sites is calculated from k_{-1} to be 0.08 - 0.14 second. As the passage time for sites in the blood through the tubular capillaries is quoted to be about 2.5 second (Best & Taylor Baltimore 1961) the results indicate that the dissociation of S from HSA is not likely to limit the renal tubular secretion rate.

Widman, M and Sandberg T (Department of Pharmacognosy Faculty 236
of Pharmacy University of Uppsala Sweden): PLASMA PROTEIN BINDING
OF 7-HYDROXY- Δ^1 -TETRAHYDROCANNABINOL: AN ACTIVE CANNABIS METABOLITE

Earlier we have shown that Δ^1 tetrahydrocannabinol the major psycho-tomimetically active compound of Cannabis is extensively bound to the lipoproteins of human plasma. 7-Hydroxy- Δ^1 tetrahydrocannabinol which is a pharmacologically and psychologically active metabolite of Δ^1 tetrahydrocannabinol in man, has been shown by equilibrium dialysis and ultrafiltration to be bound to 94-99% to plasma proteins. Further experiments using the [^{14}C] labelled compound, with agarose and polyacrylamide gel electrophoresis and ultrafiltration suggest that albumin α_1 -lipoprotein and to a minor degree also β lipoprotein are involved in the protein binding of 7-hydroxy- Δ^1 -tetrahydrocannabinol in blood plasma.

237 Aitio A (Department of Physiology University of Turku Finland)
DISTRIBUTION AND INDUCIBILITY OF UDP GLUCURONYLTRANSFERASE IN DIFFERENT RAT TISSUES

Liver has been regarded as the main site of glucuronide formation though UDP glucuronyltransferase activity has been detected also in the kidney skin and gastrointestinal mucous membrane. Recently also lung has been demonstrated to possess transferase activity (cf Aitio Xenobiotica 1973 in press). Therefore a reinvestigation of the presence and inducibility of UDP glucuronyltransferase in different rat tissues was undertaken.

A low UDP glucuronyltransferase (4-methylumbelliferone) activity was detected in the brain retroperitoneal fat and cardiac and diaphragmatic muscle and adrenal gland. An intermediate activity was found in the thymus spleen and lung. A high activity resided in the gastrointestinal mucosa kidney and liver. The blood was totally devoid of activity. A low activity was demonstrated in the placenta too. Drug induced rise of UDP glucuronyltransferase activity was studied in the kidney liver lung spleen and thymus. Treatment with cinchophen resulted in an increase of the activity in the lung liver and kidney whereas 3-methylcholanthrene caused an increase in the liver and lung only. The inducing power of phenobarbital was restricted to the liver and chlorpromazine was devoid of inducing potency.

Thus all the portals of foreign compounds to the body as well most other organs possess at least some capacity to transform xenobiotics to glucuronides which are water soluble and thus easily excreted.
Grant: U.S. Public Health Service (AM-06018-11)

Klausen M (Laboratory of Pharmacology Royal Danish School of Pharmacy Copenhagen Denmark): RAPID TRANSIENT TOLERANCE TO PENTOBARBITAL

The mice given 30 mg/kg pentobarbital i.p. show a plasma concentration of 15-20 µg/ml after 30 min. They are anesthetized and unable to run on the rotarod (J.D. Christensen Acta Pharmacol. in press). Re-administration of the same dose after 12-19 hours gives a decreased anesthesia and in some mice a nearly normal motor coordination measured on the rotarod after 30 min. The repeated dose 24 hours after the first dose shows a less pronounced tolerance than that observed after 12-19 hours. The pentobarbital plasma concentration of the mice made tolerant is correspondingly diminished so that the observed tolerance is mainly metabolic.

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ON THE TURNOVER OF ACETYLCHOLINE IN THE BRAIN

AN EXPERIMENTAL STUDY USING
INTRAVENOUSLY INJECTED RADIOACTIVE CHOLINE

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The present survey is based on the following papers and some unpublished results

- I. H. Sparf and J. Schuberth Metabolic disposition of radioactive choline in the mouse with a special reference to the transport from plasma to brain. Submitted for publication in *Eur J Pharmacol*.
- II. L. Ewetz, B. Sparf and B. Sörbo Enzymatic determination of choline in brain with choline phosphokinase and ^{32}P -labelled ATP Symposium on *in vitro* Procedures with Radioisotopes in Medicine in Vienna, 1969 Ed. International Atomic Energy Agency (1970). SM-124/39 175-183
- III. J. Schuberth, B. Sparf and A. Sundwall A technique for the study of acetylcholine turnover in mouse brain *in vivo*. *J Neurochem.* 16 (1969) 695-700.
- IV. J. Schuberth, B. Sparf and A. Sundwall On the turnover of acetylcholine in nerve endings of mouse brain *in vivo*. *J Neurochem.* 17 (1970) 461-468.
- V. S. M. Aquilonius, F. Flentge, J. Schuberth, B. Sparf and A. Sundwall. Synthesis of acetylcholine in different compartments of brain nerve terminals *in vivo* as studied by the incorporation of choline from plasma and the effect of pentobarbital on this process. *J Neurochem.* 20 (1973) 1509-1521
- VI. B. Lundholm and B. Sparf The effect of atropine on the turnover of acetylcholine in the brain. Submitted for publication in *Eur J Pharmacol*.

In the following, the investigations are cited by their Roman numerals.

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1 INTRODUCTION

Biosynthesis of acetylcholine (ACh) in brain tissue was demonstrated as early as 1936 by Quastel and co-workers by using slices from brain cortex. In 1943 Nachmansohn and Mackado showed that extracts from brain tissue could synthesize ACh in the presence of choline (Ch) under anaerobic conditions without glucose, if adenosine-5 -triphosphate (ATP) was added. The enzyme system which could synthesize ACh from Ch was named cholinacetylase. The significance of acetylcoenzyme A (acetyl-CoA) for the synthesis of ACh from Ch was described by Korkes *et al.* 1952. When subsequently it was generally accepted that the acetylation of Ch with acetyl-CoA occurred in two steps, the enzyme catalyzing the last step (see below) was called choline acetyltransferase (ChAc) (EC 2.3.1.6). This term had previously been introduced by Nachmansohn (1951).



In 1956 Hebb showed that 60 % of the ChAc activity of a brain homogenate was localized in the mitochondrial fraction after centrifugation and the rest in the supernatant. An important subsequent discovery was that the nerve terminals in the brain withstand homogenization in sucrose. They became detached but seem to remain intact in isotonic media. These nerve ending particles or synaptosomes have been isolated by differential and gradient centrifugation (Whittaker 1959). Several investigations have shown that ACh (~ 80 %) and ChAc (~ 60 %) are localized in the nerve ending fraction (Hebb and Whittaker 1958 Hebb and Smallman 1956).

Further methods were developed by de Robertis *et al.* (1963) and Whittaker Michaelson and Kirkland (1964) to isolate the vesicles, which have been observed in the nerve ending particles by electron microscopy. These vesicles, some of which contain ACh, could be released from the nerve endings by hypotonic shock. De Robertis postulated that the vesicles were both the site of synthesis and the storage place of the transmitter.

Whittaker believed that the vesicles only were the storage place of the ACh. These different opinions were based on the supposition that ChAc is localized in the vesicles (de Robertis *et al.* 1963 McCaman, de Lores Arnaiz and de Robertis 1965) or that it is soluble in the cytoplasm of the nerve ending (Whittaker 1964). Further it has been suggested that the enzyme is bound to a non-vesicular

component (Tuček 1966). Later it was shown that pH and ionic strength are of great importance for the binding properties of ChAc to the vesicles and other membrane fragments (Fonnum 1967). By extrapolating to *in vivo* conditions it was assumed that the enzyme is normally in free form in the cytoplasm.

A relatively large number of drugs with various pharmacological action have been shown to influence both the release of ACh from different cortical areas and the amount of ACh in the brain (Mitchel 1960 Bartolini and Pepeu 1967 Richter and Crossland 1949 Holmstedt, Lundgren and Sundwall 1963 Giarman and Pepeu 1962 Pepeu 1963 Consolo *et al.* 1972). However no attempts have been made yet to study the effects of drugs on the turnover of ACh in the brain. This would provide valuable information on the regulation of the synthesis, storage and release of ACh. The aim of the present investigation has been to study the turnover of ACh in the brain, particularly how pharmacological agents interfere in this process.

As the brain seems to be incapable of synthesizing Ch *de novo*, because the brain tissue cannot convert ethanolamine lipids into Ch lipids by stepwise methylation of the base (Bremer and Greenberg 1961 Marshall, Chojnacki and Ansell 1965 Ansell and Spanner 1967 1968), Ch has to be supplied to the brain from plasma. Consequently it seemed expedient to use intravenously administered labelled Ch as the precursor. It was therefore necessary to know the fate of the precursor in the plasma and to separate and identify the radioactive Ch metabolites (cf. Fig. 1) (I). To enable measurement of the specific radioactivity (SA) not only of ACh but also of Ch, much work has been done to develop methods for the determination of endogenous Ch in the brain (II-III). This made it possible to estimate the turnover of ACh in the whole brain and in the nerve endings by using intravenously injected labelled Ch (III, IV). The localization of labelled Ch and ACh in subcellular fractions of brain nerve endings was studied in paper V. The functional importance of the findings has been investigated by studying the effects of drugs, such as pentobarbital, oxotremorine and atropine, on the parameters (III-VI and unpublished results).

METABOLISM OF CHOLINE

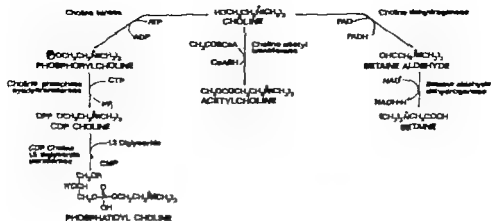


Fig. 1 Metabolic pathways of choline.

2. GENERAL APPROACH

2.1 Turnover principles

There has been considerable confusion concerning the turnover terminology. However, Zilvermit has proposed the following definitions. *Turnover rate* The quantity of material turned over per unit of time. The meaning of turnover rate is unequivocal only when a steady state exists, i.e., when the rate of synthesis and transport into a compartment equals the rate of breakdown and exit. *Turnover time* The time required for the turnover of an amount of material equal to the pool size (Zilvermit 1955).

When studying the turnover of a substance much information is obtained by establishing a precursor-product relationship. The following three criteria for an immediate precursor are most used (Zilvermit 1943).

- i) The specific radioactivity (SA) of the immediate precursor is greater than that of its product before the latter reaches its maximum SA.
- ii) After the product has reached its maximum SA, the slope of the SA-time curve of the product is negative and therefore the SA of the compound is greater than that of its precursor.
- iii) At the time when the product has reached its maximum SA the slope of the SA-time curve of the product is zero and therefore the SA of the immediate precursor equals the SA of the product at that time.

In the present study the ACh turnover in the brain has been estimated by measuring the isotopic dilution of the precursor Ch in the brain and the initial rate of the radioactive ACh formation after intravenous injection of labelled Ch. This estimation of the ACh turnover is based on some assumptions which are summarized below.

- i) The brain Ch is localized in an open single compartment system.
- ii) There is a rapid equilibration of the injected radioactive Ch with the endogenous store of Ch.
- iii) The synthesis of ACh from Ch is the rate limiting step (not the transport of Ch to the site for ACh synthesis).

All these assumptions are probably not fulfilled but the approach may nevertheless be valuable as the main intention is to use this technique to detect

changes in turnover rate of ACh induced by drugs. This turnover has also been calculated from the decay curve of the labelled ACh. However the results are influenced by concomitant synthesis of ACh. As will be described in chapter 4 there is probably also a re-uptake and re-use of radioactive Ch from released and hydrolyzed ACh. These difficulties might at least partly be overcome by blockade of ChAc but at present there is no ChAc inhibitor which functions satisfactorily *in vivo*. Therefore, this approach will result in an underestimate of ACh turnover.

Another way to investigate the effect of a drug on the ACh turnover is to inject the drug intravenously after the injection of the radioactive Ch when (^3H)-ACh has reached its maximal level in the brain. The effect on the ACh turnover could then be estimated by comparing the decay curves of (^3H)-ACh in the brains of the control and the drug treated animals (VI).

2.2 Determination of choline in the brain

The difficulties in assaying free Ch in the brain can be seen from Table I, where values of the Ch content of the brain, which have been reported in the literature are tabulated. Besides the differences in the specificities of the methods, there are other difficulties in estimating free Ch in the brain. Thus, less than 1 % of total brain Ch ($\sim 20,000$ nmoles/g) is free Ch (cf. Schubert, Sparf and Sundwall 1970 a). Further glycerophosphorylcholine (GPhCh) is present in the brain in about 400 nmoles/g (Ansell and Spanner 1970). As shown in Fig. 2, GPhCh can also be formed from both Ch plasmalogen and lecithin in animal tissue (Dawson 1966). GPhCh can split off Ch in brain tissue, a reaction catalyzed by GPhCh diesterase (Webster Marples and Thompson 1957), and it is readily hydrolyzed to yield Ch under acidic conditions (Appleton *et al.* 1953). Thus, there are possible errors due both to killing techniques (enzymatic hydrolysis before denaturation of diesterases) and to extraction procedure (acid hydrolysis due to e.g., the acid protein precipitating agent) which must be considered. A rapid postmortal increase of endogenous free Ch does in fact occur in brain tissue if the brains are not frozen rapidly enough (II IV and Dross and Kewitz 1972).

As the methods previously used for Ch estimation in tissues (e.g., before 1967 when this work started) are rather unspecific and circumstantial a new method was developed (paper III). The method, which was the first enzymatic assay for Ch is based on the ChAc catalyzed formation of labelled ACh from labelled acetyl-CoA and the Ch in the extract.



Table I
Summary of determinations of free Ch in the brain

Authors	Publishing Date	Material	Method	Content nmoles/g	(n)
Smith & Saelens	1967	Rat brain	ChAc	600	
Ansell & Spanner	1968	Rat brain	Periodide	170 \pm 10	(5)
Hebb	1968	Rat brain	Biological	200	
Marchbanks	1968	Guinea pig cortex	Biological	220	
Schubert, Sparf & Sundwall	1969	Mouse brain	ChAc	115 \pm 2.4	(5)
Ewertz, Sparf & Sörbo	1969	Rat brain	Ch kinase	39.3 \pm 0.85	(10)
Ewertz, Sparf & Sörbo	1969	Mouse brain	Ch kinase	43.0 (41.4-45.5)	
Saelens, Allen & Simke	1970	Mouse brain	ChAc	131.5 \pm 5.3	(27)
Hansen, Matarelli & Costa	1970	Rat brain	Gas chromatography	274 \pm 16	(11)
Schubert & Sundwall	1971	Mouse brain	ChAc	68.1 \pm 6.11	(10)
Reid, Haeberlich & Krishna	1971	Rat brain	Ch kinase	67.8 \pm 4.8	(7)
Codler Poon & Salehmooghadam	1972	Mouse brain	Biological	68.8 \pm 6.4	(9)
Consolo, Ladinsky Peri & Garattini	1972	Mouse brain	ChAc	62.7 \pm 2.0	(4)
Browning	1972	Rat brain	Fluorometry	107 \pm 8	(4)
Dross & Kewitz	1972	Rat brain	Biological	42	
Dross & Kewitz	1972	Rat brain	Biological	27.5* \pm 1.7	
Eade, Hebb & Mann	1973	Rat brain	Biological	21.5 \pm 5.1	(10)
Eade, Hebb & Mann	1973	Rat brain	ChAc	29.1 \pm 10.1	(11)
Eade, Hebb & Mann	1973	Rat brain	Ch kinase	35.0 \pm 11.0	(4)

* Extrapolation to *in vivo* conditions from postmortal increase of free Ch.

The labelled ACh formed was determined by radioassay after separation from the excess of labelled acetyl-CoA. While the present study was in progress the same principle was published by Smith and Saelens 1967 who found the brain to contain about 600 nmoles Ch/g of brain tissue. By our version of the ChAc method a Ch value of 115 nmoles/g was obtained (III).

At about the same time two other reports on the Ch content of the brain were published. By using a periodide method (Ansell and Spanner 1968) and a biological method (Hebb 1968), the brains were found to contain 170 nmoles/g and 200 nmoles/g respectively. Owing to the different values obtained a new principle was used (II). It is based on the Ch phosphokinase reaction and radioassay of the radioactive PhCh formed from the reaction between added $AT^{32}P$ and Ch in the extract.

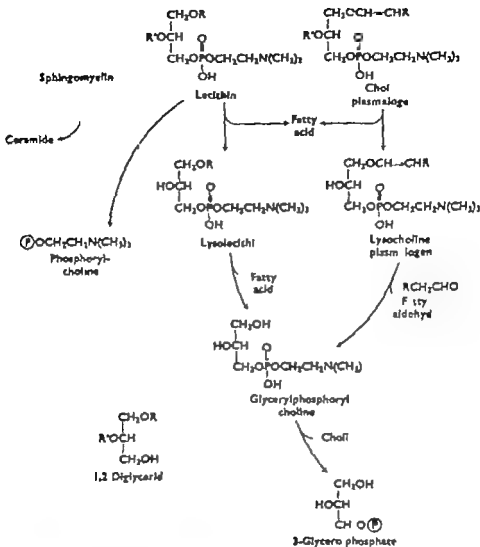


Fig. 2. Break down of choline containing phospholipids.



By this method rat brain was found to contain 39.3 ± 0.95 nmoles Ch/g and mouse brain 43.0 nmoles/g. As the above Ch values were lower than had been reported previously with other methods (Table I) a number of control experiments were performed (II).

There are, however at present also some practical disadvantages in its rather

low sensitivity and because the Ch phosphokinase is rather unstable. However smaller amounts may be estimated with a higher SA of $AT^{32}P$ (0.05 mCi/mM) or by decreasing the incubation volume (2 ml). An advantage is that the enzymatic phosphorylation reaction can be carried out under extremely mild conditions which, as mentioned, is important for the estimation of Ch in the brain.

Recently two further methods, in which Ch kinase is employed for the determination of both ACh and Ch have been published. One involves a preliminary separation of ACh and Ch by electrophoresis (Reid, Haubrich and Krishna 1971). By this method about 0.4 nmoles/g brain could be measured and the rat brain was found to contain 65 nmoles Ch/g. The other method uses reactions which are catalyzed by the following enzymes: ACh-esterase, Ch phosphokinase, pyruvate kinase and lactate dehydrogenase (Browning 1972). By this procedure 0.1 nmole of Ch and ACh could be detected and the Ch content of the brain was estimated at 106 nmoles/g. The advantage of these new assays using Ch phosphokinase, is their higher sensitivity in comparison with the original Ch phosphokinase method at present. However they also give somewhat higher values than the method described in paper (II) (cf. Table I) which seems to be one of the most thoroughly checked (II).

By modifying our original ChAc method, the Ch values decreased from 115 nmoles to 68 nmoles Ch/g. The same principle used by Consolo *et al* (1972) gives 62.7 nmoles/g.

From the discussion above it seems obvious that there is no single simple explanation for the varying Ch values reported for brain tissue. Very recently Eade, Hebb and Mann (1973) have reported a comparison of three different methods based on different principles. A biological method (determination of ACh on the rectus abdominis of the frog after acetylation of Ch in the extract with acetylchloride) was run parallelly with a modification of the Ch phosphokinase method (II) and a ChAc assay. The Ch phosphokinase method confirmed the low value obtained in paper II but the ChAc assay and the bioassay gave still lower values (29 and 22 nmoles/g. respectively).

By combining a precipitation reaction (e.g., reineckate) and gas chromatography or gas chromatography/mass spectrometry methods have been developed with a high sensitivity for determination of the SA of Ch and ACh in turnover studies with (1C) and deuterium Ch as ACh precursors (Hanin, Massarelli and Costa 1972; Jenden 1972). These new methods have probably great potential possibilities in the future but the costs may be a limitation. Methodological difficulties have been present resulting in too high Ch values of the brain (cf. Table I).

To sum up, it may be concluded that many of the values published (Table I) are too high owing to factors such as the postmortal increase in free Ch, hydrolysis of Ch containing compounds by the acid extractants or too low specificity of the methods. The exact value of free Ch in the brain is, therefore, not known, but all evidence taken together indicates a Ch value in the range of 40 ± 20 nmol/g. This value (40 nmol/g) has been used in this work for calculations of the SA of Ch in brain.

2.3 Determination of acetylcholine

The brains were homogenized in trichloroacetic acid which was removed by extraction with ether. The endogenous ACh in the extract was measured on a small strip of the dorsal muscle of the leech (Szerb 1961). When known amounts of ACh were added to the tissue fractions no potentiation or inhibition was observed. Further insignificant contractions were obtained by samples treated with alkali (pH 11–12) for 30 min at room temperature. In the presence of d-tubocurarine (10^{-5} M) less than 10 % of the original ACh-like activity of the extracts were obtained. Ch was about 1,000 times less active than ACh on the leech muscle. By the present technique 0.5–5 pmoles of ACh in a tissue extract can be determined. The mouse brain was found to contain 16.9 ± 1.52 nmol/g (mean \pm S.E. $n=6$). This value is in agreement with those of Karlén *et al.* (1973), who compared biological estimation of ACh with methods based on gas chromatography/mass spectrometry and pyrolysis gas chromatography (Karlén *et al.* 1972).

2.4 Separation of radioactive choline metabolites

For the present study an efficient separation of the Ch metabolites (Fig. 1) was essential. This was initially obtained by isolating the quaternary ammonium compounds in the brain tissue extracts by reineckate precipitation followed by exchange of the reinecke ion for chloride on a Dowex-X8 anion column. Labelled Ch and ACh was then separated by high voltage electrophoresis at pH 4.6 in 0.2 M acetate buffer (Heilbronn and Carlsson 1960). A disadvantage of this system, used in papers III and IV and partly in paper I, is that the desalting step with reineckate precipitation is rather time consuming and that PhCh is lost as it is not precipitated with reineckate.

For these reasons the high voltage electrophoresis system was modified by a buffer of the following composition pyridine:acetic acid:acetone:water

(8.8.30 154 pH 4.6) (I). Using this system Ch, ACh, PhCh, GPhCh and cytidyldiphosphorylcholine (CDPCh) can be separated in concentrated trichloroacetic acid extracts. By complementing this system with high voltage electrophoresis at pH 10 and acetylcholinesterase (AChE) hydrolysis of the ACh spot obtained at electrophoresis at pH 4.6 the following substances could be separated Ch, ACh PhCh, betaine aldehyde, betaine, GPhCh, CDPCh and lecithin. Reproducible recoveries in the range of 85–90 % were achieved for Ch, ACh, PhCh and betaine. The tailing from the Ch to the ACh spot was less than 0.5 % on electrophoresis at pH 4.6. Since complex binding of TCA to quaternary ammonium compounds has been reported to influence the migration of Ch-metabolites in chromatography systems (McCarthy Knight and Chenoweth 1973), the recoveries of the different Ch metabolites were carefully checked during different conditions (I and III).

3 TRANSPORT OF CHOLINE FROM PLASMA TO BRAIN

3.1 The fate of choline in plasma

Free Ch in the brain is probably used as the precursor of both ACh and phospholipids. However as previously mentioned, the brain appears unable to synthesize Ch *de novo* and should therefore be dependent upon an exogenous supply of Ch.

The only known pathway for *de novo* synthesis of Ch in higher animals is the stepwise methylation of phosphatidylethanolamines by adenosylmethionine to form phosphatidylcholine (lecithin) (Bremer and Greenberg 1960). This route was shown to be of significance for the biosynthesis of lecithins in the liver (Björnsdahl and Bremer 1966). Estimates by Wise and Elwyn (1965) indicate that this pathway may provide Ch in amounts equivalent to the dietary intake of the rat, or about 13 μ moles per day and gram liver. Lecithins may also be formed by incorporation of preformed free Ch via PhCh and CDPCh (Fig. 1 Kennedy and Weiss 1956). That the latter route is mainly responsible for the formation of lecithin in the liver has been suggested by Haimes (1966).

For the reasons mentioned above, intravenously injected radioactive Ch was thought to be useful for studying the turnover and compartmentation of ACh in the brain. Consequently it is of importance to know the fate of the injected labelled Ch also in plasma. In the literature there is some evidence that the plasma concentration of Ch is remarkably constant around 10 nmoles/ml and that it is neither changed by exercise or by a meal, nor greatly affected by various liver or kidney diseases (Bligh 1952). Moreover when exogenous Ch is injected into the plasma, the increased Ch level is rapidly corrected to a normal value (Bligh 1953). However a more detailed evaluation of the time course of the metabolic disposal of Ch when administering high doses or tracer amounts of Ch has not previously been made. As seen in paper I, doses of 500 and 2,000 nmoles given intravenously produced in 2 sec a marked increase of the Ch plasma level (147 and 581 nmoles/ml). Despite a negligible urinary excretion of radioactivity the plasma concentration was normal (10–15 nmoles/ml) after 5 and 20 min respectively. After injection of 10 nmoles Ch no increase in plasma Ch was observed and the biological half-life in plasma was about 5 min.

Most of the injected radioactive Ch was recovered as PhCh in tissues (jejunum, liver and brain). The responsible enzyme (Ch kinase) has been found in the brain (highest activity), stomach, kidney, intestinal mucosa, liver and heart (McCaman 1962). Apparently most organs of the mouse seem to be able to convert Ch to PhCh. The latter can be further metabolized in the organs, via the Kennedy pathway to lecithin (Fig. 1).

From findings in paper I it seems evident that the oxidation of Ch to betaine in most tissues is a minor pathway in comparison with the phosphorylation of Ch. This is contrary to suggestions made by Bligh (1953) who proposed that the Ch oxidation was the major pathway. The importance of the oxidation step is apparently to generate methyl groups, as betaine is a better methyl donor than Ch (Borsook and Dubnoff 1947). However, betaine is one of the major metabolites in plasma after (^3H)-Ch injection. This betaine must be carried to the plasma from tissues as plasma is not able to metabolize Ch *in vitro* (Sparf unpublished results). On the other hand, no labelled PhCh is found in the plasma which is the major metabolite in most tissue. This is due to hydrolysis of PhCh by enzymes present in plasma, which was observed after intravenous injection of labelled PhCh (I). Labelled GPhCh or CDPCh could not be found in the plasma after intravenous injection of labelled Ch. The appearance of labelled phosphatidylcholine in plasma was very slow (I).

3.2. The source of free choline in the brain

Experiments with the perfused cervical ganglion in the rat have shown that the Ch concentration in the perfusion medium is of great importance for optimal ACh synthesis during synaptic activity (Burks and MacIntosh 1961, Collier and MacIntosh 1969). It also appears that about half the Ch formed from released ACh is immediately recaptured and resynthesized into ACh (Collier and MacIntosh 1969).

In general, investigations have shown that Ch is transported to the tissues by a low affinity uptake mechanism, e.g. kidney (Rennik 1958, Sung and Johnstone 1965, 1969), giant axons of *Loligo* (Hodgkin and Martin 1965), erythrocytes (Martin 1968), brain cortex slices (Schubert *et al.* 1966 a, Schubert Sundwall and Sörbo 1967), synaptosomes (Marchbanks 1968 b, Potter 1968, Diamond and Kennedy 1969, Abdel Latif and Smith 1972) and microsomal, synaptosomal, mitochondrial and synaptic vesicle fractions of rat brain (Diamond and Millay 1972).

Is there a similar transport mechanism of free Ch from plasma into brain or is Ch first converted into a more lipid soluble form. The latter alternative has been suggested by Ansell and Spanner (1971). The postulated mechanism is based on the finding that from one to six hours after intraperitoneal injection of labelled Ch, ethanolamine, dimethylaminoethanol or methionine, lipids in the liver and brain became radioactive "whereas no labelled Ch was detectable in the blood or the brain". However, the biological half lives of radioactive Ch in the plasma and the

brain vary between 5 and 10 min (I). Therefore, in the time interval (0–60 min) incorporation of labelled free Ch in the plasma into the brain lipids could have occurred. Thus, in view of the long interval (1 and 6 hours) in the above experiment, it is not justified to consider lipid Ch in plasma as the main precursor of brain Ch. Another indication that Ch is not mainly transported to the brain as lipid-Ch is that, the fatty acid composition of brain and liver phosphatidylcholine is quite different (Ansell and Spanner 1971). Moreover their demonstration of a precursor-product relationship between CDPCh and phosphatidylcholine after *intra-cerebral* injection of labelled Ch speaks against phosphatidylcholine in plasma as the main Ch source for brain. If phosphatidylcholine is transported to a substantial extent from the plasma to the brain, then this would result in a lower SA of phosphatidylcholine compared with that of CDPCh.

Ch liberated from PhCh or phospholipid-Ch, does not seem to be an important source of Ch for ACh synthesis under physiological conditions in perfused superior cervical ganglion of the rat (Collier and Lang 1969). On the contrary as previously mentioned, free Ch must be supplied to the perfusion medium for optimal ACh synthesis.

The first evidence, for free Ch in plasma being a precursor of brain Ch, was shown in experiments in which methyl labelled Ch was injected intravenously into mice (III). As soon as 15 sec after the injection, about 15 % of the radioactive Ch, captured by the brains of conscious mice, are in the form of ACh. The SA time curve for Ch in the brain rapidly crosses that of ACh at a point where the SA of ACh was maximal, which suggests a direct precursor-product relationship (Fig. 3). The only radioactive, acid-soluble Ch-metabolites present during the time interval 0.25–20 min are Ch, ACh and PhCh (I). It seems improbable that within this short time interval the Ch has been converted to phospholipid Ch before entering the brain. The previously described time course of the metabolite pattern in the plasma and brain after intravenous injection of labelled Ch (I) is another evidence in favour of plasma Ch as the immediate precursor of brain Ch.

By injecting two different doses of Ch containing the same amount of radioactivity it was demonstrated that the relative amount of labelled Ch incorporated in the brain is decreased slightly dose dependent, if a non-tracer dose (750 nmoles) of radioactive Ch was injected instead of a tracer dose (10 nmoles). Moreover incorporation of Ch into the brain, after a 3 minute infusion, was three times more effective than after a pulse injection of the same tracer dose, as can be seen in Table II. From these findings it seems probable that there is a transport of free Ch from the plasma to the brain, which is mediated by a carrier which is saturated already at physiological-plasma Ch levels (I).

Diamond (1971) obtained evidence in support of a carrier mediated transport

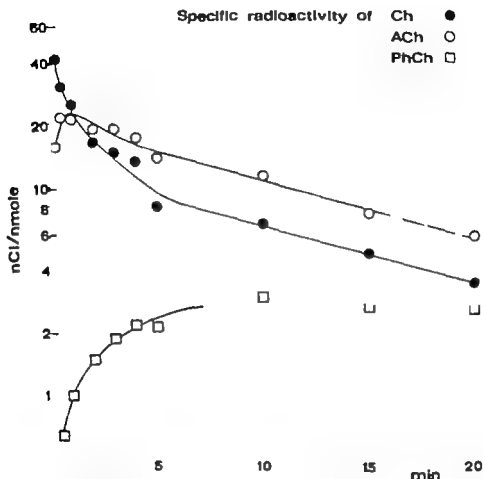


Fig. 3 The specific radioactivities of choline, acetylcholine and phosphorylcholine in the brain at different times following intravenous injection of 160 μCi (10 nmol) (^3H)-choline. Endogenous values used: Ch 40 nmol/g; ACh 15 nmol/g (ref. See text); PhCh 380 nmol/g (Arnold and Spatner). Each point expresses the mean of 3–8 experiments.

of Ch from the plasma to the brain, which is saturated at Ch concentrations ten times the normal serum levels. This difference in saturation level may be explained by the findings of Yamamura and Snyder (1972). They found that synaptosomes isolated from rat brain accumulate Ch by two kinetically distinct processes, a high affinity uptake system ($K_m=10^{-6}\text{ M}$) and a low affinity system ($K_m=10^{-4}\text{ M}$). The high affinity uptake system was associated with a consider

Table II

A comparison of the transport into brain of radioactive Ch following an intravenous pulse injection and a 3 min infusion. 160 μ Ci (3 H)-Ch (10 nmoles) were administered at zero time to mice by injection or infusion. The animals were killed 1, 3 and 5 min after the injection and 3 min after the start of the infusion. The values of the radioactive Ch and its metabolites are expressed as the mean \pm S.E., n = the number of experiments.

Route of administration	Time min	n	(3 H)-tot.	(3 H)-PhCh	(3 H)-Ch	(3 H)-ACh
I.v. injection	1	3	29.9 \pm 1.2	7.0 \pm 0.4	15.7 \pm 1.6	4.5 \pm 0.5
	3	5	34.5 \pm 1.6	16.8 \pm 0.9	13.2 \pm 1.5	6.5 \pm 0.4
	5	3	31.1 \pm 1.2	19.7 \pm 2.9	9.6 \pm 0.2	5.5 \pm 0.2
Infusion	3	6	75.3 \pm 10.6	14.3 \pm 1.4	38.2 \pm 6.3	8.3 \pm 0.8

able formation of ACh, and was assumed to represent selective Ch accumulation by cholinergic neurons. This indicates that the low-affinity uptake system was studied by Diamond as only non-tracer doses of Ch were used at the intravenous injection.

Recently a third hypothesis concerning the origin of the brain has been published (Dross and Kewitz 1972). They suppose that phosphorylated derivatives of ethanolamine (phosphorylethanolamine, CDP-ethanolamine) can be methylated in the brain to form the respective Ch derivatives. These new aspects of the origin of Ch in the brain are not compatible with the generally held view (see above) that the brain cannot synthesize Ch *de novo*. The hypothesis as advanced by Dross and Kewitz (1972) is based on the following findings

- The concentration of free Ch in the brain increases at a velocity of about 20 nmoles/g and min immediately after decapitation. This is assumed to be due to the cessation of venous drainage while normal Ch synthesis proceeds.
- An arteriovenous difference was observed between the Ch concentration of the femoral artery (12 ± 0.5 nmoles/ml) and that of the jugular vein (19.4 ± 1.7 nmoles/ml).
- After intravenous injection of (14 C)-Ch the peak of the SA of Ch in the brain is reached in less than 1 min but amounts to only one tenth of the SA of Ch in plasma. There is a parallel decline of the SA in both compartments.

From these findings they conclude that Ch readily penetrates the blood-brain barrier and that the concentration gradient is directed from the brain to the venous blood. Ch is continuously formed in the brain, which process continuously

dilutes the labelled Ch, and prevents the SA in the blood and the brain from becoming equal. An alternative explanation would be that there is a more rapid synthesis of ACh and PhCh from labelled Ch in the brain compared with the transport rate of labelled Ch from the plasma to the brain. If this is the case, one of the assumptions, previously mentioned, for estimating the turnover of ACh by measuring the initial formation of labelled ACh in the brain, is probably not fulfilled. As a consequence, this would result in a too low value of the turnover.

After intravenous injection of labelled Ch the SA of PhCh in the brain did not reach the SA of Ch (Fig. 3 Dross and Kewitz 1972). Although Ch is certainly a first order precursor of PhCh the finding indicates that PhCh reaches the pool also via an alternative pathway e.g., phosphorylation of ethanolamine or hydrolysis of compounds containing PhCh. The lower SA was used by Dross and Kewitz as an additional argument in support of the existence in the brain of a methylation pathway. However in metabolic systems in which the product compartment is not homogeneous, the SA curves of precursor and product will necessarily not cross (Zilversmit 1960). This might occur in the present case, as a synthesis of labelled ACh proceeds simultaneously with a synthesis of labelled PhCh.

In short, the conclusion of the present author is that, free Ch seems to be transported to the brain from the plasma via a carrier-mediated, transport mechanism which is saturated at physiological plasma levels of Ch (1). This process is connected with a rapid synthesis of ACh in the brain. It seems as if a minor part of the brain Ch may be the result of a lipid-Ch transport, as an arterio-venous difference of Ch has been demonstrated. There is no direct evidence yet that Ch can be synthesized *de novo* in the brain.

4 TURNOVER OF ACETYLCHOLINE IN THE BRAIN AND ITS NERVE ENDINGS

4.1. Estimation of turnover rates of acetylcholine

The rate of formation and the rate of disappearance of the radioactive ACh formed in the brain from intravenously injected labelled Ch, have been used to determine the turnover rate of ACh in the brain. By measuring both the initial rate of formation of labelled ACh and the isotopic dilution of the labelled Ch in the brain the turnover rate was estimated at 50 nmoles/g brain/min (III). This calculation was based on a 40 % conversion of labelled Ch to ACh per g of brain and min, and on an endogenous Ch value of 115 nmoles/g (III). However as discussed earlier a Ch content of about 50 nmoles/g brain is more probable, which would decrease the calculated turnover rate to about 20 nmoles/g/min.

The turnover study in paper III can be criticized since it was performed with a dose of (^3H)-Ch of 500 nmoles. However as seen in Table III very similar results were obtained with a dose of 10 nmoles of Ch which leaves the plasma level completely unchanged. When the timecourse of the SA of Ch and ACh in the whole brain is followed, a precursor product relationship could be rather well established, as previously mentioned (Fig. 3). Objections can be raised against the calculation of turnover rates by the present method, as the estimation is based on the assumptions that the brain Ch is localized in one pool, and that the injected labelled Ch is rapidly equilibrated with the endogenous Ch pool. How valid are these assumptions?

The ratio labelled Ch:ACh was rather similar (about 2:1) in both the whole

Table III

The conversion of (^3H)-Ch to (^3H)-ACh in the brain after a pulse injection intravenously of 500 nmoles of (^3H)-Ch and 10 nmoles of (^3H)-Ch.

The values are expressed as the mean of ((^3H)-ACh as % of (^3H)-Ch) \pm S.E., n = number of experiments.

Time (min)	(^3H)-Ch (500 nmoles) (^3H)-ACh/(^3H)-Ch(%)	n	(^3H)-Ch (10 nmoles) (^3H)-ACh/(^3H)-Ch(%)	n
0.25	10.4 \pm 1.32	4	13.4 \pm 0.98	6
0.50	15.1 \pm 1.15	5	24.9 \pm 0.83	7
1	24.2 \pm 0.10	2	30.0 \pm 2.00	8
2	37.4 \pm 0.72	2	41.0 \pm 2.01	3
5	38.9 \pm 0.45	2	59.6 \pm 2.49	4
10	46.1 \pm 1.05	2	63.3 \pm 3.28	4

brain and the isolated nerve endings during the time interval 2–20 min after intravenous injection of Ch (IV). The half-lives of both Ch and ACh were also approximately the same (7 min) in both the whole brain and the nerve endings (IV). Since radioactive and endogenous ACh is almost exclusively localized in the nerve endings, the findings indicate that also radioactive Ch is mainly localized in the nerve endings. This means that the assumption mentioned in the general approach concerning a model of an open single compartment system for the calculation of turnover can be used. The fact that the ratios mentioned become equal already after 2 min points to a rather rapid mixing of radioactivity with the endogenous pools.

A further support for an immediate, main transport of Ch in the plasma to the nerve endings is the previously mentioned, high affinity uptake of Ch by the synaptosomes of the brain *in vitro* which is associated with considerable ACh synthesis (Yamamura and Snyder 1972). The rather similar half life of Ch in plasma and Ch and ACh in the brain may also be an indication of a rapid equilibration of plasma Ch with brain Ch. The existence of a specific Ch uptake by cholinergic nerve endings is indicated by experiments performed by Kuhar *et al.* (1973). They found that the Ch uptake at 10^{-5} to 10^{-6} M by synaptosomes *in vitro* was reduced by lesions only in brain regions in which a high cholinergic input is probable. These results also speak in favour of the hypothesis that cholinergic nerve endings are responsible for the bulk of the free Ch accumulated by the brain tissue.

Since mouse brain contains about 15 nmoles/g of ACh (13 nmoles/g, Crossland and Merrich 1954, 16 nmoles/g, Consolo *et al.* 1972) the estimated turnover rate would mean that the brain renews its ACh store about every minute. This rate lies between the turnover rate found in the perfused cervical ganglion (10 nmoles/g/min) and the initial restitution rate of ACh in the brain after partial depletion of the ACh store by electro-shock (40 nmoles/g/min) (MacIntosh 1963, Richter and Crossland 1949). The latter value is rather near the maximal synthesis rate obtainable *in vitro* by ChAc from 1 g mouse brain tissue (60 nmoles/g/min) (Schnier and Shuster 1967).

Another approach to calculate the ACh turnover in the brain is also possible. The size of the Ch pool that is used for ACh synthesis can be calculated by using the ratio 2:1 between the labelled Ch and the labelled ACh in the nerve endings 2 to 20 min after intravenous injection of labelled Ch (IV). If about 80 % of the endogenous ACh in mouse brain (15 nmoles/g) are in the nerve endings (Hebb and Whittaker 1968), and 50 % of the ACh in the nerve endings are located in the cytoplasm (Chakrin and Whittaker 1969) the Ch concentration in the synaptosomal cytoplasm (compartment for ACh synthesis?), calculated on the

ratio 2:1 between labelled Ch and ACh, would correspond to about 12 nmoles/g brain. However this calculation according to pool size was based on the assumption that the incorporation of ACh into the synaptic vesicles is very slow (Chakrin and Whittaker 1969 Marchbanks 1969). This means that most of the radioactive ACh formed during this short time interval is in the cytosol compartment. However as shown in paper V there is probably a very rapid incorporation of radioactive ACh into a high molecular weight fraction of the nerve endings. As will be discussed in the next paragraph it also seems as if it might be a very intimate coupling, between the synthesis and storage of ACh, to a high molecular weight constituent in the nerve ending. This would indicate that the ACh pool in the nerve ending may still be regarded as an open single compartment and then consists of up to 12 nmoles ACh/g. From the ratio labelled Ch/labelled ACh (2:1) the Ch pool could be estimated at 25 nmoles/g. The turnover rate of ACh could then be calculated at about 10 nmoles/g/min (40 % initial conversion of labelled Ch to labelled ACh/g/min).

A third method which is a more speculative way to calculate the turnover was used in paper IV. Based on calculations of the volume of the nerve endings (Marchbanks 1968 b) and kinetic data of ChAc (Schubert 1966) the rate of ACh formation at the estimated concentrations of the substrates was calculated at about 10 nmoles ACh/g/min (IV). Since the optimal synthesis rate is 60 nmoles ACh/min obtainable *in vitro* by ChAc from 1 g mouse brain (Schrier and Shuster 1967) instead of 160 nmoles/min, which was erroneously cited in paper IV this value is probably about 5 nmoles ACh/g/min.

An often used way to estimate the turnover of catecholamines is to study the decay curves of the radioactive labelled transmitter after blocking its synthesis. The disappearance curve of labelled ACh from the brain may reflect not only the turnover of ACh but also other factors such as a continuing synthesis from the remaining labelled Ch, and the re-uptake to the nerve endings of Ch from released and hydrolyzed, labelled ACh. In ganglia half the Ch formed from released ACh seems to be recaptured and resynthesized to ACh (Collier and MacIntosh 1969). The ACh turnover (~ 1 nmole/g/min) calculated from the half-life ($t_{1/2} \sim 7$ min) of labelled ACh is about twenty times lower than the turnover estimated from the initial formation of labelled ACh (20 nmoles/g/min). The half-lives of labelled Ch and ACh in the brain may also be influenced by the Ch half-life in plasma. There is about the same half-life of free Ch in the plasma and the brain (I).

It is not possible to calculate the synthesis rate from the changes in the SA of the precursor and the product in the plasma and the brain when (3 H)-Ch was pulse injected. This is owing to the SA of Ch in plasma being higher than that of the brain during the whole time interval studied. The latter approach has been used for

determining the synthesis rate of dopamine, noradrenaline and serotonin in the brain stem (Neff *et al* 1971).

However by infusing (^3H)-Ch into rats and following the SA of Ch and ACh in the brain, and assuming an open single compartment model, the efflux rates of ACh have recently been determined in the cortex, midbrain and brainstem (Saelens *et al* 1973). In the cortex the rate was 20 nmoles/min/g. which is in good agreement with the turnover rate found in the whole brain of the mouse when applying our technique, and confirmed by Saelens *et al* 1973 who used the same method.

A valuable pharmacological tool, when studying the ACh turnover by means of the declining ACh curve, would be a centrally active ChAc inhibitor. Unfortunately the inhibitors available today are unsuitable for this purpose (Aquilonius *et al* 1971) as has been previously mentioned.

From the above discussion, it is obvious that a number of assumptions must be made in order to be able to calculate the turnover of ACh in the brain. It has not been possible to ascertain to which extent these assumptions are valid. However by using two different ways to calculate the ACh turnover rate, rather similar values were obtained 10–20 nmoles ACh/g/min).

4.2 Compartmentation of newly synthesized acetylcholine

As previously mentioned endogenous ACh in the nerve endings is distributed in at least two morphological compartments, namely in the cytoplasm and in the vesicles (de Robertis *et al* 1963 Whittaker *et al* 1964). The incorporation of radioactive ACh into different pools of the nerve endings has been studied by Chakrin and Whittaker (1969) by injecting radioactive Ch intracortically into anaesthetized cats and guinea pigs. These experiments showed a slow incorporation of the synthesized transmitter into the vesicles as compared with the cytosol. Similar results were obtained when the conversion of radioactive Ch to ACh was studied in the nerve endings of the brain *in vitro* (Marchbanks 1968 b Richter and Marchbanks 1971). Indirect evidence of an additional small ACh pool with a high turnover was obtained when Ch was injected intraventricularly into conscious guinea pigs under local anaesthesia (Barker *et al* 1970). These results were obtained by a comparison of the SA of synaptosomal ACh at different stages of the isolation procedures. Thus the pool was readily lost when synaptosomes were separated, on a sucrose density gradient, from the other constituents of the crude mitochondrial fraction (P_2 in Fig. 4), myelin and mitochondria.

Intracortical injections seem rather unphysiological especially since in the

PREPARATION OF A HIGH (HMWF) AND LOW (LMWF) MOLECULAR WEIGHT FRACTION FROM BRAIN NERVE ENDINGS

Homogenization of a mouse brain in 0.3 M sucrose containing 10^{-4} M eserine

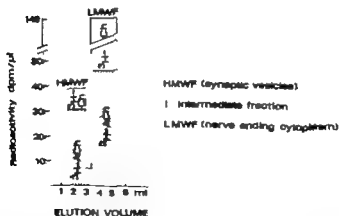
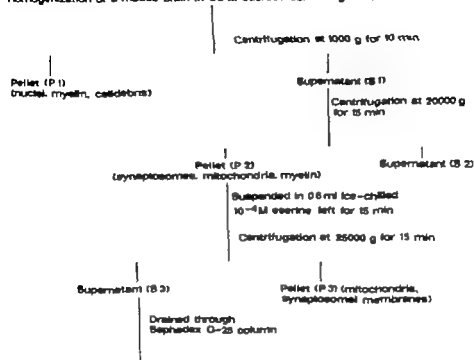


Fig. 4 Subcellular fractionation of a high (HMWF) and a low (LMWF) molecular weight fraction from a mouse brain. The mouse was injected intravenously with 566 μ Ci (H)-Cb and killed by blow on the neck three min later. The homogenization of the brain was started within 30–45 sec after death.

present study there are indications, that plasma Ch is the physiological precursor of brain ACh. Therefore, the incorporation of labelled ACh into the different pools of the nerve endings was studied by using the present *in vitro* technique. The content of lysed synaptosomes was separated into a high (HMWF) and a low (LMWF) molecular weight fraction by a modification of the current gel filtration procedures (Marchbanks 1968 b Kadota and Kanaseki 1969).

In our experiments the lysed, nerve ending fraction was first centrifuged, and mitochondria and membrane fragments were pelleted before passing the supernatant through a Sephadex G 25 fine column. By eluting the column with 10 mM of sodium chloride two distinct fractions could be obtained. The whole isolation procedure of the HMWF and the LMWF which, in this way is comparatively rapidly performed, is shown in Fig. 4

The intention was to follow the SA of both ACh and Ch in these two fractions, in order to elucidate the compartmentation of ACh and the site of its biosynthesis in the nerve ending. After intravenous injection the incorporation of radioactive ACh into both the LMWF and HMWF is very rapid (V) Already 15-30 sec after the injection, the incorporation of both labelled Ch and ACh were maximal in the two fractions. An interesting finding is that the ratio of (^3H)-ACh/(^3H)-Ch was about 10 times higher in the HMWF than in the LMWF

When the SA of ACh was calculated, a higher SA of ACh was found in the HMWF than in the LMWF contrary to the findings of Chakrin and Whittaker (1969) who reported that the cytoplasmic fraction of ACh showed a SA which was twice that of the vesicular. However the estimations were done only at one time interval and, as late as 60 min after an intracerebral injection of labelled Ch. Furthermore the animals were anaesthetized with a barbiturate, which decreases the ACh turnover in the brain (III).

Our study (V) demonstrated that the SA of ACh was significantly higher in the HMWF in 18 of 21 experiments, in the interval 0.25-15 min after the intravenous injection of labelled Ch. In two of the three experiments where the SA of ACh in the LMWF was higher than in the HMWF this was observed at 15 min. Owing to the rapid incorporation of labelled Ch and ACh, no distinct maximum could be established at the times studied. Therefore, it was not possible to establish a precursor-product relationship from the kinetics of the SA of ACh in the HMWF and the LMWF

The SA of ACh in the two fractions is not known in the time interval 0-0.25 min but two possibilities exist

- i) the SA of ACh is higher in the HMWF than in the LMWF also during this period. In that case synthesis

a) proceeds independently in the two compartments but at a higher rate in HMWF. In fact a more rapid half life of the SA of ACh in the HMWF ($t_{1/2} \sim 7$ min) compared with that of the LMWF ($t_{1/2} \sim 12$ min) is indicated in the time interval 1–15 min after the (3 H)-Ch injection.

b) Alternatively synthesis occurs only in the HMWF but a leak of labelled ACh from HMWF to LMWF occurs during the isolation procedures.

c) If the product compartment (in this case ACh in the LMWF) is not homogeneous, the two SA curves will not cross each other (Zilvermit 1960). The latter explanation is likely if the ACh synthesis occurs in the HMWF but the LMWF consists of a two- or multi-compartment system.

ii) The SA of ACh in the LMWF crosses that of the HMWF at an extremely early stage, indicating a primary synthesis in the LMWF

To further elucidate the site of the ACh biosynthesis, the SA of Ch were estimated in the two fractions. However immediately after homogenization, a value of 185 nmoles Ch/g was found. On the assumption that the yield of the endogenous Ch is the same as that of the labelled Ch in the P_2 fraction i.e. 11 % (V), the Ch concentration in this fraction should be about 5 nmoles/g. The actual value found was, however 41 nmoles/g. Therefore, the concentration found in the lysed P_2 is probably too high. No increase in the amount of Ch occurs during chromatography of the lysed P_2 on the Sephadex column. Incubation experiments indicate that the Ch concentration in the HMWF remains unchanged during the fractionation procedure (V). Consequently the Ch concentration found in this fraction may correspond to *in vivo* conditions. The considerably lower SA of Ch, than that of ACh in the HMWF at all the times investigated, indicates that ACh is probably not synthesized from the Ch within the HMWF. On the other hand, the concentration of Ch in the LMWF *in vivo* may possibly be about seven times lower (5 nmoles/g instead of 36 nmoles/g) than indicated in paper V.

If this is true the SA of the Ch in the LMWF (presumably corresponding to cytoplasmic Ch) would, at least at the shortest time-intervals, be higher than that of the ACh in the HMWF (presumably corresponding to vesicular ACh). For these reasons cytoplasmic Ch is the most likely immediate precursor of vesicular ACh. A possible hypothesis in explanation of the findings is that, the ACh biosynthesis occurs in the cytoplasmic compartment, but in close association with synaptic vesicles or another high molecular constituent. Using an *in vitro* system, Ritchie and Goldberg (1970) were able to demonstrate an incorporation of ACh into a vesicle fraction only in the presence of concomitant ACh synthesis. Conse-

quently a direct coupling seems plausible between the biosynthesis and the binding of ACh to a constituent present in the vesicular fraction.

This hypothesis will also explain the failure to demonstrate an uptake of ACh by isolated vesicles *in vitro* (Marchbanks 1968 a) and the experiments by Katz, Salehmoghaddam and Collier (1973) which showed that ACh taken up by nerve endings cannot be released by electrical stimulation of the nerve. It has also been demonstrated that the amount of exogenous ACh accumulated by a cervical ganglion is small when compared with the amount of ACh synthesized from exogenous Ch (Collier and MacIntosh 1969).

A puzzling finding in our study (V) was the low yield of (^3H) ACh in the lysed fraction P_2 (28 %) as compared with the corresponding recovery of endogenous ACh (60 %). A somewhat higher yield of labelled ACh (37–41 %) in the isolated P_2 fraction was obtained in a former study (IV). A still higher yield of endogenous ACh in the P_2 fraction of 70 % was found by Whittaker (1959).

This unexpected discrepancy between the recovery of labelled and of endogenous ACh could be due to a more labile form of binding of newly synthesized ACh than of preformed ACh. This interpretation is also in agreement with results obtained by Barker *et al* 1970.

The discrepancy could be explained by populations of nerve endings from some regions of the brain with a high ACh turnover resulting in a large proportion of newly synthesized transmitter or by a heterogeneous vesicle population in the nerve ending. After labelling the ACh store under physiological conditions, the functionally most active vesicles, which are presumably close to the presynaptic membrane may incorporate ACh of a higher SA than those vesicles which are more centrally located and probably more inactive. The latter hypothesis of heterogeneous vesicles is supported by a report from Roth *et al* 1968 who isolated two populations of vesicles containing norepinephrine ACh containing vesicles with different characteristics have also been isolated from guinea pig brain (Kadoya and Kanaseki 1969).

To further elucidate the compartmentation of newly synthesized ACh in different nerve ending and vesicle populations, experiments for studying ACh turnover in different brain regions and vesicle fractions are now in progress.

5 EFFECT OF DRUGS ON THE TURNOVER OF ACETYLCHOLINE

As mentioned in the introduction the amount of ACh in the brain changes when the functional activity of the organ is altered by certain drugs. For example, the administration of pentobarbital and oxotremorine is associated with an increase in the ACh content whereas administration of e.g., atropine is associated with a decrease (Giarman and Pepeu 1962, Holmstedt, Lundgren and Sundwall 1963, Consolo *et al.* 1972). Further it has been demonstrated that barbiturates decrease and atropine increases the release of ACh from the cerebral cortex (Mitchel 1960, Bartolini and Pepeu 1967). However by studying the effect of these drugs on the ACh turnover in the brain more knowledge should be obtained concerning the regulation of the synthesis, storage and release of ACh. Consequently the effects of a barbiturate (pentobarbital), a cholinergic (oxotremorine), and an anticholinergic drug (atropine) on the turnover have now been studied by means of the present *in vivo* techniques.

When the effect of pentobarbital on the initial rate of formation of labelled ACh was studied, it was found that the turnover rate was diminished to 1/4 of its normal value (II). As the drug causes a reduction in the body temperature of mice, this has also been compensated for by keeping the mice at 38°C. As shown in Fig. 5 the reduced rate of synthesis of radioactive ACh is due, only to a minor degree, to the hypothermia (Schuberth, Sparf and Sundwall 1970 b).

During anaesthesia the radioactive Ch in the brain was found to be about 10–20% higher than in conscious mice. Hence, one could suspect that the reduced rate of ACh synthesis reflects an overall decrease in Ch metabolism. Therefore, experiments were performed in which the major products of Ch metabolism in the brain were measured. It was observed that the biosynthesis of both the labelled PhCh and the phospholipids was much less affected than that of ACh (Fig. 6). This indicates that the effect of pentobarbital on ACh synthesis is rather specific. This specificity was also found to be dose related (Fig. 7) (Schuberth, Sparf and Sundwall, to be published).

Oxotremorine, at an intraperitoneal dose of 0.5 mg/kg, which was found to be the threshold dose for tremor also diminished the ACh turnover rate in the brain to 1/4 (III). The effect occurred even when the decrease in the body temperature of the mice was inhibited (Fig. 8). This effect on turnover may be compared with earlier findings which showed that the increased ACh levels in rat brain produced by oxotremorine (Holmstedt, Lundgren and Sundwall 1967), are not mediated by a fall in body temperature (Campbell, Hamm and Jenden 1969). Oxotremorine also had a dose dependent effect on the ACh turnover. Uptake of radioactivity

Relationship between ^3H -ACh and ^3H -Ch in mouse brain at different times after injection of ^3H Ch

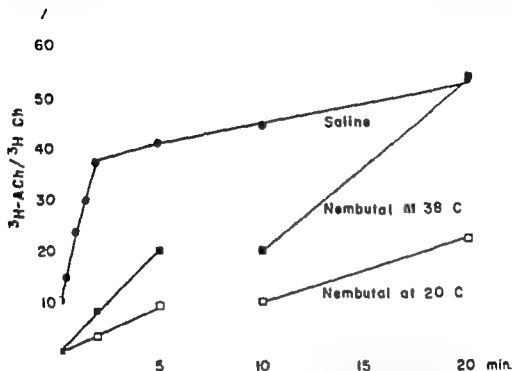


Fig. 5 Synthesis of radioactive ACh in mouse brain *in vivo*. 100 μCi (^3H) Ch (500 nmoles) were given intravenously at zero time, and the ratio, radioactive ACh/radioactive Ch, was analyzed at different times. The mice were killed by immersion in liquid nitrogen. Pentobarbital (60 mg/kg) was injected intraperitoneally 30 min before the injection of (^3H) Ch. In one group of the pentobarbital treated mice, hypothermia was prevented by warming the animal at 38°C. Each point represents the mean of 4 experiments.

and incorporation of Ch into PhCh and phospholipids were only slightly affected (Fig. 9). Contrary to the findings in the present investigation *in vivo*, oxotremorine increases the ACh synthesis in cerebral cortex slices *in vitro* (Howes *et al.* 1970). The explanation for these different results, which were obtained *in vitro* and *in vivo*, is not known.

Oxotremorine and pentobarbital increase the ACh level and decrease the ACh turnover *in vivo*. However, probably different mechanisms are involved. This is indicated by the increase in the Ch levels produced by oxotremorine, contrary to pentobarbital (III and Consolo *et al.* 1972).

FATE OF ^3H -CHOLINE IN MOUSE BRAIN

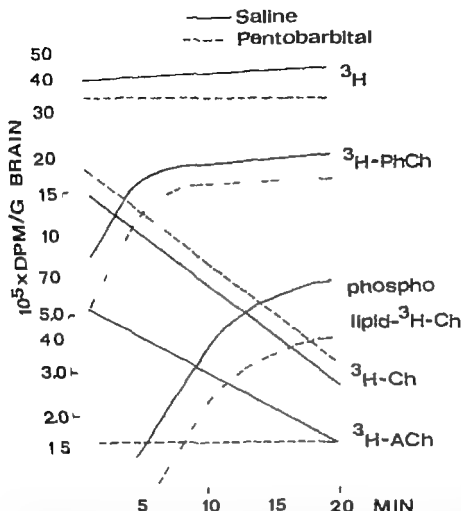


Fig. 6. Effect of pentobarbital on the uptake and metabolism of (^3H)-Ch in mouse brain *in vivo* (^3H -total radioactivity). 160 μCi (^3H)-Ch (10 nmol) were injected intravenously 30 min after intraperitoneal injection of pentobarbital (60 mg/kg). The mice were killed in liquid nitrogen at different times after the injection of (^3H)-Ch. Hypothermia was prevented by warming the mice at 38°C . Each point represents the mean of 4 experiments.

Atropine at an intravenous dose of 5 mg/kg had no measurable effect on the initial rate of ACh formation (VI). Therefore its possible effect on the exponential decline of (^3H)-ACh in the brain was investigated. Atropine was injected intra

FATE OF ^3H -CHOLINE IN MOUSE BRAIN

Pentobarbital

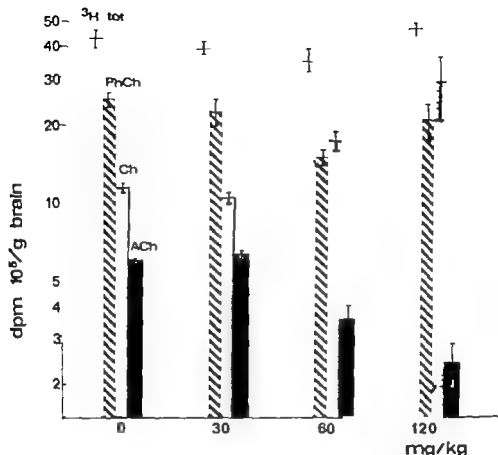


Fig. 7 The effect of different doses of pentobarbital on the uptake and metabolism of (^3H) Ch in mouse brain *in vitro*. (H)-tot. = total radioactivity 160 μCi (H)-Ch (10 nmol) were injected intravenously 30 min after intraperitoneal injection of pentobarbital. The mice were killed in liquid nitrogen 5 min after the injection of (^3H)-Ch. Mean \pm S.E. is indicated, $n=2-3$.

venously 3 min after the injection of labelled Ch, when the labelled ACh had reached its peak value in the brain. It was observed that atropine decreased the half-life of labelled ACh to ~ 5 min compared with a half-life of ~ 10 min in the control group (VI). The SA of ACh did not change 2–17 min after the atropine injection. No significant change in the time curves of labelled Ch, PhCh or total radioactivity occurred.

The increase in the ACh turnover indicated by the more rapid half-life of (^3H)-

Relationship between ^3H -ACh and ^3H -Ch in mouse brain at different times after injection of ^3H -Ch

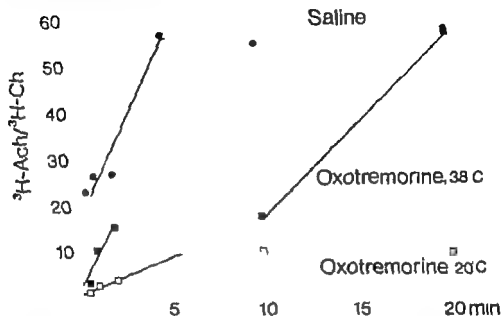


Fig. 8. Synthesis of radioactive ACh in mouse brain *in vivo*. $160 \mu\text{Ci } (^3\text{H}) \text{Ch}$ (10 nmoles) were given intravenously at zero time, and the ratio radioactive ACh/radioactive Ch in whole brain was analyzed at different times. Oxotremorine (1 mg/kg) was given intraperitoneally 30 min before the injection of $(^3\text{H}) \text{Ch}$. Each point represents the mean of 2 experiments.

ACh in the brain probably reflects a higher release of ACh from the nerve endings. Many types of experiments have indicated, that it is most recently stored or synthesized transmitter which is preferentially released (superior cervical ganglion, Collier 1969; diaphragm, Potter 1970; splenic nerve, Kopin *et al.* 1968; caudate nucleus, Benson *et al.* 1969). The experiments in paper VI are not readily consistent with the hypothesis that the most recently synthesized transmitter is the most readily released. This may be explained by the fact that the release of ACh after atropine treatment differs in this respect, e.g. that all ACh pools are equally affected. However more experiments are needed to clarify this.

The effect of pentobarbital was also studied after the injection of $(^3\text{H})\text{-Ch}$. When injecting a dose of 40 mg/kg of pentobarbital intravenously 3 min after the injection of labelled Ch, the endogenous ACh had increased about 50 % already 2 min after the pentobarbital injection. At that point of time the mice had lost their

FATE OF ^3H -CHOLINE IN MOUSE BRAIN

Oxotremorine

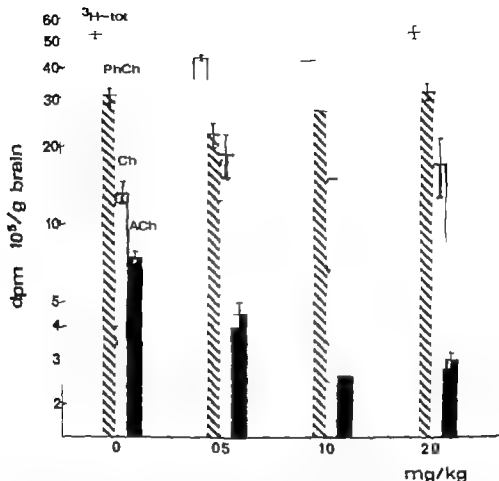


Fig. 9 The effect of different doses of oxotremorine on the uptake and metabolism of (^3H) Ch in mouse brain *in vitro*. (H)-tot. = total radioactivity. $160 \mu\text{Ci}$ (^3H)-Ch (10 nmoles) were injected intravenously 30 min after intraperitoneal injection of oxotremorine. The mice were killed in liquid nitrogen 5 min after the injection of (^3H)-Ch. Mean \pm S.E. is indicated, $n=2$.

righting reflex and the reflex was lost throughout the observation period (20 min). As shown in Fig. 10 the half life of (^3H)-ACh is increased ($t_{1/2} \sim 20$ min) after injection of pentobarbital, while the curves for (^3H)-PhCh and total radioactivity were not affected at all. During preliminary experiments it was found that, contrary to the controls, the SA of ACh is just slightly changed, during the time

FATE OF ^3H -CHOLINE IN MOUSE BRAIN

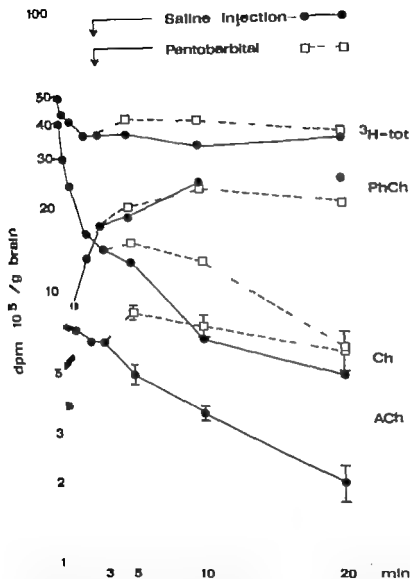


Fig. 10. The effect of pentobarbital on the metabolism of (^3H) -Ch in the brain. Pentobarbital (40 mg/kg) or saline were injected intravenously 3 min after intravenous injection of $160 \mu\text{Ci } (^3\text{H})$ -Ch (10 nmoles). The values of (^3H) -ACh are expressed as mean \pm S.E. Each point is the mean of 4-8 experiments. Vertical bars represent \pm S.E.

interval studied, following pentobarbital, which, at least superficially regarded, indicates a reduced release of newly synthesized ACh.

A complicating factor when studying the effect of drugs on the ACh turnover in whole brain is the possible regional differences. That such regional effects exist has been demonstrated by Nordberg and Sundwall, 1973. Differences in the effect of drugs on the ACh turnover on the subcellular level must also be taken in consideration.

When the effect of pentobarbital on the compartmentation of ACh in the nerve endings was studied, the most prominent effect was the markedly reduced incorporation of (3 H)-Ch into the (3 H)-ACh into the HMWF. The incorporation into the LMWF was affected to a much smaller extent. The endogenous ACh in the HMWF was only slightly increased, which agrees with the findings of Kurokawa *et al.* (1963) and of Beani *et al.* (1969). They found an increase in labile bound ACh (cytoplasm?) whereas the stable bound ACh (vesicles?) remains relatively unchanged.

Is there any plausible explanation of the decrease in ACh turnover and the increase of endogenous ACh in the LMWF following pentobarbital? An inhibition of ChAc in rat brain by ACh *in vitro* has been demonstrated by Katta and Goldberg (1969). They found that ACh, at a concentration of 10 mM inhibited its own synthesis by 10 % and that inhibition is progressively increased to 45 % at a concentration of 100 mM ACh. If there is a product inhibition of ChAc also *in vivo* a local increase of the ACh concentration in the low molecular weight compartment could lead to a decrease in the ACh synthesis there. However after injection of pentobarbital the most pronounced effect on synthesized, labelled ACh was in the high molecular weight compartment. The increase in endogenous ACh in the presumed cytoplasmic compartment after pentobarbital indicates that the regulation of the rate of ACh synthesis might occur in the cytoplasmic compartment, and that synthesis is intimately connected with the storage mechanism. The pentobarbital effect indicates also that the binding of ACh to a vesicular constituent may be of direct functional importance.

Another attractive theory is that it may be the concentration of the precursors acetyl-CoA or Ch which regulates the ACh synthesis. That the level of acetyl-CoA is significantly decreased by oxotremorine at a dose of 1 mg/kg but not at 0.5 mg/kg has been shown by Schubert *et al.* 1966 b. This finding is, however difficult to interpretate as only about 5 % of the amount of acetyl-CoA in the brain takes part in the ACh formation. Atropine (100 mg/kg) and pentobarbital (30 mg/kg) did not change the acetyl-CoA level.

That the Ch concentration can be proposed as a regulatory mechanism of the ACh synthesis is based upon the fact that there is high-affinity uptake of Ch to

synaptosomes, which is connected with a rapid synthesis of ACh. However both the simultaneous decrease in ACh turnover with an increase in Ch level following oxotremorine, and the increase in ACh turnover and unchanged level of Ch following atropine do not support the latter theory

To further elucidate the mechanisms which regulate the synthesis, storage and release of ACh, the effect of drugs on the turnover of ACh in different brain regions and subcellular fractions, will be studied. Such experiments are now in progress.

ACh in the brain is synthesized from acetyl-CoA and Ch. The latter does not seem to be synthesized in the brain *de novo* (Bremer and Greenberg 1961 Ansell and Spanner 1967 1968). More probably Ch is transported from plasma to the brain as free Ch (I III), presumably by a carrier mediated mechanism saturated already at physiological plasma levels of Ch (I). However Ch may also be transported to the brain as lipid-Ch (Ansell and Spanner 1971), which is indicated by an arterio-venous difference between the Ch level in the femoral artery and jugular vein observed by Dross and Kewitz 1972. In the brain Ch seems to be rapidly equilibrated with the Ch in the nerve endings (IV). The transport of free Ch is associated with a rapid synthesis of ACh (III) which has also been shown in synaptosomes *in vitro* when they accumulate Ch by a high affinity uptake mechanism (Yamamura and Snyder 1973). Hence, plasma Ch seems to be the immediate, major precursor of ACh in the brain in addition to the Ch split off from brain lecithin and the re-uptake of Ch from released and hydrolyzed ACh.

The level of free Ch in the brain seems to be in the range 40 ± 20 nmoles/g (II and Eade, Hebb and Mann 1973) about 2–3 times the ACh level. This broad Ch range may be due to too low specificities of some of the present methods, hydrolysis of phospholipids by acidic protein precipitating agents but also post mortal increase of Ch (II and IV).

By using labelled Ch, intravenously injected into mice the turnover rate of ACh in the brain has been calculated at 10–20 nmoles/g/min. Similar results have recently been obtained by Saelens for the rate of ACh efflux in the cortex of rats (20 nmoles/g/min) found by infusion of (3 H)-Ch (Saelens *et al.* 1973). Drugs like pentobarbital and oxotremorine decrease the ACh turnover in the brain (III Schubert Sparf and Sundwall 1970 b). Both drugs increase the ACh level in the brain (Giarman and Pepen 1962 Holmstedt, Lundgren and Sundwall 1963) but pentobarbital, contrary to oxotremorine, does not seem to increase the Ch level or decrease the acetyl-CoA level in the brain (III Consolo *et al.* 1972 Schubert *et al.* 1966 b), indicating that different mechanisms are involved.

ACh synthesized from labelled Ch *in vitro*, is very rapidly incorporated into a high molecular weight fraction, isolated from lysed synaptosomes. The pronounced decrease in the incorporation of ACh in the high molecular weight fraction, presumably a vesicular fraction caused by pentobarbital, indicates that the binding of ACh to a vesicular constituent may be of direct functional importance (V). By comparing the SA of Ch and ACh in the presumed isolated cytoplasmic and the vesicular fraction, it is postulated that the ACh synthesis

occurs in the cytoplasm but is closely associated with the binding of ACh to a constituent, present in the vesicular fraction (V). The hypothesis will also explain the failure to demonstrate an uptake of ACh by isolated vesicles *in vitro* (Marchbanks 1968 a) and the experiments by Katz *et al.* (1973) which showed that ACh taken up by nerve endings cannot be released by electrical stimulation of the nerve.

6. SUMMARY

ACh in the brain is synthesized from acetyl-CoA and Ch. The latter does not seem to be synthesized in the brain *de novo* (Bremer and Greenberg 1961 Ansell and Spanner 1967 1968) More probably Ch is transported from plasma to the brain as free Ch (I, III), presumably by a carrier mediated mechanism saturated already at physiological plasma levels of Ch (I). However Ch may also be transported to the brain as lipid-Ch (Ansell and Spanner 1971), which is indicated by an arterio-venous difference between the Ch level in the femoral artery and jugular vein observed by Dross and Kewitz 1972. In the brain Ch seems to be rapidly equilibrated with the Ch in the nerve endings (IV). The transport of free Ch is associated with a rapid synthesis of ACh (III) which has also been shown in synaptosomes *in vitro* when they accumulate Ch by a high affinity uptake mechanism (Yamamura and Snyder 1973). Hence, plasma Ch seems to be the immediate, major precursor of ACh in the brain in addition to the Ch split off from brain lecithin and the re-uptake of Ch from released and hydrolyzed ACh.

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**HISTOCHEMICAL AND ELECTRON
MICROSCOPIC OBSERVATIONS ON THE
DEVELOPMENT, NEURAL CONTROL AND
FUNCTION OF THE PANETH CELLS
OF THE MOUSE**

FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI,
SILTAVUORENPENGER 20, HELSINKI 17 FINLAND

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 398

**HISTOCHEMICAL AND ELECTRON MICROSCOPIC
OBSERVATIONS ON THE DEVELOPMENT
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HELSINKI 1973

PREFACE

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Antti Abonen

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INTRODUCTION

The pyramidal cells at the bottom of the small intestinal crypts have been first described by Schwalbe (1872) and a rather profound reinvestigation at the light microscope level was made later by Paneth (1888). First electron microscope investigation of these cells was made by Hally (1958).

It has been estimated that average human intestine contains about 200 000 000 Paneth cells (Toner 1968). The number of Paneth cells increases in some pathological conditions (Lauren 1961 Toner 1968 Otto and Fett 1972).

Although Paneth cells may secrete enormous quantities of enzymes into the intestinal fluid the precise nature of the secretion is still unknown. Nor it is known if the secretion of the Paneth cells is under neural or hormonal control and at what age the secretion begins. It is not known what role the Paneth cells have in the pathological conditions where they appear in the human stomach or colon (Lauren 1961 Toner 1968 Otto and Fett 1972).

REVIEW OF THE LITERATURE

Distribution of the Paneth cells

Paneth cells are present in about 50 % duodenal and 75 % of ileal and jejunal crypts (Toner 1968) and they are always situated at the bottom of the crypts and granule containing Paneth cells have never been seen to be higher up in the villi (Deschner 1967 Cheng et al 1969 Troughton and Trier 1969).

Paneth cells are present in man the mouse rat, guinea pig (Taylor and Flaa 1964) and ant bear (de Castro et al. 1952) rabbit (Pitha 1968) and some other species (Schaaf and Wenzel 1970) but no Paneth cells have been found in the cat, dog and birds (Taylor and Flaa 1964).

Morphology of the Paneth cells

Paneth cells are pyramidal cells which have microvilli in their apical parts projecting into crypt lumen. Nucleus of the cells is often irregular and nucleolus is well developed (Hally 1958 Kurosuni 1961 Behnke and Moe 1964).

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(Deschner 1967 Cheng et al. 1969 Troughton and Trier 1969 Browning and Trier 1969) However the cells die and a new cell replaces the old the generation time being about 3 weeks (Cheng et al. 1969) Paneth cells possibly originate from less differentiated intermediate cells (Cheng et al. 1969 Sperschneider et al. 1972)

Function of the Paneth cells

It has been assumed that the function of the Paneth cells is to secrete digestive enzymes into the intestinal lumen (Hally 1958) especially peptidases (Behnke and Moe 1964 Kurosuni 1961) to secrete heavy metals (Halbhuter et al. 1970) to phagocytose bacteria (Erlandsen and Chase 1972 a, b) and to secrete amino acids (Gent and Creamer 1972) These or similar cells are found in the stomach and the colon under some pathological conditions (Lauren 1961 Toner 1968 Otto and Fett 1972) Lipids and proteins in the intestinal lumen have been shown to stimulate the secretion of the Paneth cells (Hally 1958) Milk diet increases the number of Paneth cells significantly (Recknagel et al. 1972)

Effect of drugs on the Paneth cells

Pilocarpine stimulates the secretion of the Paneth cells (Stanley and Trier 1965 Trier et al. 1967 Ahonen 1970) whereas atropine inhibits it (Stanley and Trier 1965 Trier et al. 1967) The Paneth cells of the mouse are able to take up catecholamines or their precursors (Penttilä and Ahonen 1969 Ahonen and Penttilä 1969 1970 1971) This uptake can be intensified by monoamine oxidase inhibition and prevented by imipramine and cocaine (Ahonen and Penttilä 1970 1971) Paneth cells are also able to take up labelled leucine (Stanley and Trier 1965 Trier et al. 1967) glycine methionine cysteine and tryptophan (Merzel 1967) glucose and arginine (Halbhuter et al. 1972) and heavy metals (Halbhuter et al. 1970) A total protein synthesis inhibition for two weeks decreases the number of Paneth cells and the number of granules per cell but is not capable of abolishing the typical secretory granules from the cells (Eisenhuth and Geyer 1966)

PURPOSE OF THE PRESENT STUDY

Present study was undertaken to resolve

- whether the Paneth cells were under neural sympathetic and parasympathetic control
- at what time does the normal Paneth cell secretion begin
- whether there are cells related to small Intestinal Paneth cells in the mouse colon
- whether there is decarboxylase activity in the Paneth cells of the mouse
- which cells exhibit ortho-phthalaldehyde induced fluorescence in the intestine of the mouse.

GENERAL MATERIAL AND METHODS

The material consisted of 317 albino mice of both sexes of an randomly bred strain used in the Department of Anatomy. The age of the experimental animals was from 5 days to six weeks. The animals were fasted 24 hours before killing but they were allowed to drink tap water ad libidum.

Histological methods

For histological methods tissue samples were fixed with 3.5 % formaldehyde in 0.1 M phosphate buffer pH 7.2 dehydrated embedded in paraffin wax and the sections were stained with Best's carmine (Lauren et al. 1967) and Thioflavine T (Burns and Whitehead 1966). For other histological staining reactions the standard histological methods were used. Thioflavine T stained sections were viewed with fluorescence microscope (see below). All the other histological sections were viewed with the usual light microscope.

Fluorescence histochemical methods

For fluorescence histochemical methods the mice were fasted one day before the experiment and they were given nisalimide (Nisamid[®] Pfizer) 500 mg/kg body weight intraperitoneally and about one hour later dopamine

chloride (Orion) 160 mg/kg intraperitoneally. The drugs were dissolved in physiologic saline solution. The animals were killed at different intervals after dopamine injection. In each group there were 3—4 mice. Specimens were taken from the duodenum, jejunum, ileum and colon, frozen in isopentane precooled with liquid nitrogen, freeze-dried in vacuo at -40°C in the presence of phosphorus pentoxide for two days, heated to room temperature in vacuo for 4 hours and thereafter kept in vapour derived from paraformaldehyde at 80°C for one hour. The relative humidity of the formaldehyde vapour was equilibrated to 60 %. The specimens were embedded in Epon, sectioned with LKB Pyramitome, mounted in entellan and viewed with Leitz Ortholux fluorescence microscope provided with BG 3 and BG 12 and TAL 405 excitation and 470 nm barrier filters (for ref. see Eränkö 1967).

Electron microscopic methods

For electron microscopy 2—3 small tissue pieces were taken from different parts of the colon and fixed with ice cold 3.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 2 hours and postfixed with 1 % osmium tetroxide in 0.1 M phosphate buffer after which they were rinsed with phosphate buffer, embedded in Epon Araldite, sectioned with LKB ultratome, poststained with uranyl acetate and lead citrate and viewed with AEI 801 electron microscope.

Histochemical reactions

To control vagotomy histochemical acetylcholinesterase and non-specific cholinesterase methods (Koelle 1951) were used. To demonstrate acetylcholinesterase activity acetylthiocholine iodine (Fluka) was used as substrate and iso-OMPA (10^{-6} M) as inhibitor and to demonstrate non-specific cholinesterase butyrylthiocholine iodine was used as substrate and 1,5-bis-(4-allyldimethyl-ammoniumphenyl)pentan-3-one-di iodine (10^{-6} M) as inhibitors respectively.

The term *Secretion* means in the present investigation the disappearance of the secretory granule from the apical cytoplasm of the Paneth cell into the crypt lumen.

PURPOSE OF THE PRESENT STUDY

Present study was undertaken to resolve

- whether the Paneth cells were under neural sympathetic and parasympathetic control
- at what time does the normal Paneth cell secretion begin
- whether there are cells related to small intestinal Paneth cells in the mouse colon
- whether there is decarboxylase activity in the Paneth cells of the mouse
- which cells exhibit ortho-phthalaldehyde induced fluorescence in the intestine of the mouse

GENERAL MATERIAL AND METHODS

The material consisted of 317 albino mice of both sexes of an randomly bred strain used in the Department of Anatomy. The age of the experimental animals was from 5 days to six weeks. The animals were fasted 24 hours before killing but they were allowed to drink tap water *ad libitum*.

Histological methods

For histological methods tissue samples were fixed with 3.5 % formaldehyde in 0.1 M phosphate buffer pH 7.2, dehydrated, embedded in paraffin wax and the sections were stained with Best's carmine (Lauren et al. 1967) and Thioflavine T (Burns and Whithead 1966). For other histological staining reactions the standard histological methods were used. Thioflavine T stained sections were viewed with fluorescence microscope (see below). All the other histological sections were viewed with the usual light microscope.

Fluorescence histochemical methods

For fluorescence histochemical methods the mice were fasted one day before the experiment and they were given nialamide (Niamid[®] Pfizer) 500 mg/kg body weight intraperitoneally and about one hour later dopamine

cells visible in the small intestine in contrast to the colon where such cells were numerous at that time (Fig. 1 and 2) Some eosinophil and lymphoid cells were also visible in the crypts at the age of 2—3 weeks (Fig. 5)

By electron microscopy the Paneth cells were demonstrable in the small intestine of the mouse after the first week of development at same time when the intestinal crypts began to develop. Intestinal villi had already developed some time earlier. At the age of 1.3 weeks the Paneth cells reached their normal adult morphology showing the characteristic electron-dense secretory granules, well-developed Golgi complexes and abundant rough endoplasmic reticulum (Fig. 7 and 8). The first Paneth cells were often long and narrow and consisted of many large mitochondria of different shapes (Fig. 8 and 9). There were regularly also some small electron dense bodies in the Golgi region (Fig. 7 and 9). The small early Paneth cell granules had a wide regular halo which disappeared when the granules had grown (Fig. 7 and 9). The diameter of the Paneth cell granules was 1—3 microns and the older Paneth cells had larger granules (Fig. 7 and 9).

During the first 3 weeks of development the Paneth cells of the mouse did not take up dopamine but started to do so at the age of 3 weeks (Fig. 6). This dopamine uptake reached its adult level at the age of 4 weeks.

Discussion

The results of the present work are in agreement with the earlier observations that Paneth cells of the mouse appear at about the age of 1 week after the birth (Merz 1967; Sperschneider et al. 1972). In the rat these cells appear about 1 week later (Toner 1968). According to the present work the appearance of the Paneth cells into the small intestine took place about at the same time as the beginning of the crypt development, which may be considered as a support of the hypothesis proposed by Creamer (1967) that Paneth cells are responsible for the normal morphology of the small intestine.

It has been proposed that Paneth cells originate from the slender intermediate cells of the crypt which take up labelled thymidine within some hours in contrast to Paneth cells which take up label only some days after the single injection (Cheng et al. 1969; Troughton and Trier 1969; Recknagel et al. 1972). These intermediate cells may possibly also become transformed into the goblet cells (Merz and Leblond 1969). However the precise origin of the Paneth cells remains obscure in the present work.

Dopamine taken up by the Paneth cells of the mouse seems to be rather tightly bound to the granules and disappears from the cells into the intestinal lumen when the whole granule is secreted (Ahonen and Penttilä 1971). It is possible that dopamine uptake and secretion of the granule marks the process of granule synthesis and secretion, which thus can be visualized by formaldehyde induced fluorescence due to dopamine. This accumulation and secretion of dopamine is similar in function of time to that received with labelled leucine (Trier et al. 1969) and glucose but different from that received with labelled arginine (Halbhauer et al. 1972). Thus the beginning of the dopamine uptake by the Paneth cells of the mouse at the age of 3 weeks can possibly be interpreted as the beginning of the normal function of the Paneth cells. The dopamine uptake reached its normal adult level at the age of 4 weeks which may reflect the normal adult secretion capacity of the Paneth cells. Simultaneously with the development of the uptake mechanism also adrenergic nerve fibers appeared into the intestine (Mirkov 1972) as well as the first lymphocytes at the age of 3 weeks (Bäck 1970 1972).

II PANETH CELLS OF THE NORMAL COLON OF THE MOUSE

Introduction

Paneth cells are able to phagocytose bacteria (Erlandsen and Chase 1972 a, b) and their granules contain lysosomal enzymes (Riecken and Pearse 1966) and lysozyme (Decker et al. 1967, Ghos and Ventrappen 1971). Under zinc free diet bacterial invasion into the Paneth cells takes place (Otto and Weiss 1972).

There is some controversy about the presence of Paneth cells in a normal colon. Instead they appear rather regularly into the human colon during ulcerative colitis (Otto and Fett 1972). The purpose of the present work is to study the presence of the Paneth cells in the normal colon of the mouse.

Material and methods

The material consisted of 26 adult albino mice of both sexes. For histological, electron microscopical and fluorescence histochemical methods the samples were taken from different parts of the colon and treated according to methods described in details in general material and methods.

Results

Characteristics of the basally located epithelial cells in the colonic crypts of the mouse were poorly visible with the usual staining methods (Fig. 10 and 11). Neither was it possible to differentiate the basal epithelial cells from goblet cells accurately by Best's carmine or iron hematoxylin techniques. Best identification of the basally located epithelial cells which were called colonic Paneth cells in the present work was obtained by staining with Thioflavine T and fluorescence microscopy after formaldehyde fixation. This method stained also the goblet cells (Fig. 12, 13, 14 and 15) and it was therefore sometimes difficult to identify the colonic Paneth cells. The granules of the colonic Paneth cells stained strongly with periodic acid-Schiff technique.

In the freeze-dried Epon sections stained with toluidine blue dark stained granular cells appeared higher up in the crypts (Fig. 16). These dark granulated cells were distinctly granular and seemed to be slightly different from the goblet cells.

In the colon of the adult non-treated mouse there was no formaldehyde-induced fluorescence (FIF) except in nerve fibers and enterochromaffin and mast cells. After nielamide and dopamine injection, strongly fluorescent, granular cells appeared at the bottom of the colonic crypts of the mouse. (Fig. 17, 18, 19 and 20). The size of the fluorescent granules varied greatly (Fig. 19 and 20). The fluorescence disappeared from the colonic epithelial cells within 3—4 hours and this loss was probably due to secretion of fluorescent granules into the cryptal lumen. The coliformic bacteria of the large bowel seemed to be rather near the basal epithelial cells but they never invaded these cells (Fig. 21).

By electron microscopy the basal epithelial cells in the colon of the mouse were observed to be distinctly different from the small intestinal Paneth cells. The granules were pale and there were more granules per cell than in the small intestine. There were also some smaller and darker granules in these cells (Fig. 21). The basal epithelial cells had a great nucleus and a well developed Golgi apparatus and a darkly stained endoplasmic reticulum. Single ribosomes could not be seen in the endoplasmic reticulum so that it is difficult to say whether the endoplasmic reticulum had a smooth or rough surface but the universal strong staining may indicate the presence of the rough endoplasmic reticulum like in the small intestinal Paneth cells (Fig. 21).

The ability of the Paneth cells in the small intestine of the mouse to take up L-dopa and dopamine was first described by Penttillä and Ahonen (1969). This accumulation of dopamine can be intensified by monoamine oxidase inhibition and prevented by cocaine and imipramine (Ahonen and Penttillä 1969, 1970, 1971). According to the present study both the Paneth cells of the small intestine and the epithelial cells at the bottom of the colonic crypts normally have no histochemically demonstrable catecholamines but take up dopamine becoming distinctly visible by FIF. The ability of the cells to take up monoamines and their precursors has been proposed possibly to reflect the neural crest origin of the cells (Pearse et al. 1969). Epithelial cells at the bottom of the colonic crypts were morphologically slightly different from the small intestinal Paneth cells but had the same capacity to take up dopamine. Therefore it is proposed that Paneth cells of the small intestine and the basal epithelial cells in the colonic crypts are embryologically related and, accordingly the basal epithelial cells in the colonic crypts can be called colonic Paneth cells. Cells resembling the small intestinal Paneth cells appear into the human colon during ulcerative colitis (Otto and Fett 1972). According to the present study the number of colonic Paneth cells per surface area is greatest at the taenial region (Fig. 13). Ulcerations in the ulcerative colitis are also often situated at the taenial region (Boyd 1961).

PART TWO

III EFFECT OF VAGOTOMY ON THE PANETH CELLS OF THE MOUSE

Introduction

It has been assumed that Paneth cells secrete digestive enzymes and that fat and protein in the intestinum stimulates the secretion of the Paneth cells (Hally 1938). Milk diet doubles the number of the Paneth cells (Recknagel et al. 1972). Paneth cells have a high basal secretion rate impossible to be disturbed significantly by normal diet (Trær et al. 1967). They can phagocytose bacteria (Erlandsen and Chase 1972 a, b) and they can take up catecholamines (Penttillä and Ahonen 1969). This uptake can be intensified with niplamide or pargylline and it is prevented by imipramine and cocaine (Ahonen and Penttillä 1970, 1971). The Paneth cells can also take up cysteine and tryptophan (Merzel 1967). Pilocarpine stimulates the

secretion of the Paneth cells (Stanley and Trier 1965 Trier et al 1967 Ahonen 1970) and atropine inhibits it (Trier et al. 1967)

In the present study the effect of bilateral vagotomy and dopamine on the Paneth cells of the mouse was studied, since vagotomy can be expected to influence the Paneth cells but no previous study is available dealing with this subject.

Material and methods

Total 90 adult albino mice of both sexes were used in the present experiment. Vagotomy was made by dissecting and removing both the anterior and the posterior trunks of the vagus nerves at the oesophagus a little above the cardia under ether anaesthesia. The animals were killed 16–24 hours later by cervical dislocation and the samples were taken from the middle part of duodenum and the mid jejunum

For fluorescence histochemical methods the mice were given intraperitoneally nialamide (Niamid[®] Pfizer) 500 mg/kg and 1 hour later dopamine chloride (Orion) 160 mg/kg intraperitoneally. The animals were killed 15, 30 and 60 min., 2, 3, 4, 5 and 6 hours after dopamine injection in both vagotomized as well as the control groups. In each group there were 3–4 mice. The specimens were treated for histological and fluorescence histochemical methods according to the standard methods described in details in general material and methods

The number of the secretory granules per cell was counted in a total of 600 duodenal and jejunal Paneth cells. The calculation in each group was made in 4 different animals and 6 adjacent 7 μ thick sections. No thickness correction was made, because the intention was to obtain relative figures rather than the exact number of granules per cell. The reliability of the calculation method was tested by making two different controls at an interval of one month, and between these controls there was no significant difference. Student *t* test was used in statistical analyses

Vagotomy was controlled by histochemical acetylcholinesterase and non-specific cholinesterase methods (Koelle 1951). After vagotomy the acetylcholinesterase positive nerve fibers disappeared from the muscle, from the submucous layer as well as from the crypt basement membrane where they were in close relationship with the crypt basement membrane in the control group (Fig 26, 27 and 28). In the control group there was a positive non-specific cholinesterase reaction in the nerve fibers near the crypt basement membrane (Fig 26) and also in the Paneth cells and the other epithelial cells. The non-specific cholinesterase reaction became stronger in the nerve cells in the submucosa after vagotomy

Results

According to the present work the secretory granules of the Paneth cells of the mouse showed a weak *masked metachromasia* (Fig 22) Normally the Paneth cell granules did not have formaldehyde-induced fluorescence (FIF) but they were well visible with Thioflavine T fluorescence instead (Fig 24) After nialamide (500 mg/kg) and dopamine (160 mg/kg) the green fluorescence appeared in the apical secretory granules within 10 minutes (Fig 23) The number of fluorescent granules increased thereafter (Fig 25) reaching its maximum about 1 hour later The secretion of the fluorescent granules into the intestinal lumen began about 1 hour after the dopamine injection and all the fluorescent granules left the Paneth cells from 3 to 4 hours after the dopamine (Table I) After vagotomy the fluorescent granules left the Paneth cell about 1 hour later (Table I)

The effect of vagotomy on the Paneth cells of the mouse was also studied by calculating the average number of granules per cell before and one day after vagotomy and it was observed that vagotomy increased the average number of granules per cell from 12.1 ± 3.2 to 13.8 ± 3.3 in the duodenum and from 8.0 ± 1.8 to 12.8 ± 3.0 in the jejunum (Fig 30 and 31) Both differences were significant ($p < 0.001$) After vagotomy the Paneth cell granules were also a little bigger with a large halo than in the control group (Fig 29) Dopamine decreased the granule content both in the duodenum and in the jejunum significantly ($p < 0.001$) The decrease was from 12.1 ± 3.2 to 9.1 ± 2.6 in the duodenum and from 8.0 ± 1.8 to 6.6 ± 1.6 in the jejunum (Fig 30 and 31)

The average number of granules of the duodenal Paneth cells was significantly ($p < 0.001$) greater than the number of granules in the jejunal cells (Fig 30) The duodenal Paneth cell granules had a slightly higher turnover time than the jejunal cell granules (Table I) A conclusion may be drawn from the calculations of granules that dopamine by decreasing the number of granules per cell significantly stimulates the secretion of the Paneth cells and had also influence in the results in Table I This stimulating effect was however similar in the experiment as well as the control groups and thus the results are comparable with each other In reality the basal secretion rate in the Paneth cells of the mouse was therefore, a little slower than the results in Table I

Discussion

Pilocarpine stimulates and atropine inhibits secretion of the Paneth cells when studied by labelled leucine (Stanley and Trier 1965 Trier et al. 1967) The present results with vagotomy further supports the idea that secretion

stimulus is mediated thorough parasympathetic, cholinergic vagus nerves. The present results that dopamine stimulates the secretion may indicate that sympathetic stimulation perhaps exerts a regulatory effect on the secretion of the Paneth cells.

The turnover time of the Paneth cell granules reported in this paper with dopamine is very similar to those obtained with glucose and leucine (Halbhuber et al. 1972 Trier et al. 1967) but differs distinctly from that obtained with arginine (Halbhuber et al. 1972) arginine appears in the Paneth cells much later after the injection and first becomes visible in the paranuclear region much below the Golgi apparatus. It therefore seems likely that dopamine, glucose and leucine may reflect active uptake and synthesis in the Paneth cells whereas arginine is possibly stored as such or bound to some other substance outside the granules and is possibly used for the synthesis of the granules later.

The close relation of the cholinergic nerves with the crypt basement membrane is similar to that observed by Pack et al. (1967) and is similar to that observed in the gastric crypts (Luck et al. 1968). Atropine and vagotomy inhibits secretion of the hydrochloric acid by the parietal cells (Hirschowitz et al. 1972 Emils 1973). According to the present results vagotomy has similar effects on the Paneth cells as on the gastric parietal cells.

TABLE No I

Effect of vagotomy on the secretion of the Paneth cells of the mouse

Dopamine fluorescence was estimated at different intervals after nialamide (500 mg/kg) and dopamine (160 mg/kg) injection intraperitoneally in the Paneth cell granules of the mouse.

Explanations: — = no fluorescence, + = trace of fluorescence,
++ = weak fluorescence +++ = moderate fluorescence,
and +++++ = strong fluorescence.

Time after dopamine	15 min	30 min	1 h	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs
VAGOTOMY								
duodenum	+	++	+++	++++	+++	++	+	—
jejunum	+	++	+++	++++	+++	+++	+	—
CONTROL								
duodenum	++	+++	++++	++	+	—	—	—
jejunum	+	+++	++++	+++	++	+	—	—

IV EFFECT OF CHEMICAL SYMPATHECTOMY WITH 6-HYDROXY DOPAMINE ON THE PANETH CELLS OF THE MOUSE

Introduction

Paneth cells are more numerous in the jejunum and the ileum than in the duodenum (Toner 1968) whereas duodenal Paneth cells have more granules (Chapter III). It has been shown by electron microscopy that Paneth cells secrete their granules into the intestinal lumen where they dissolve rapidly (Hally 1958 Kurosumi 1961 Behnke and Moe 1964).

Pilocarpine (Stanley and Trier 1965 Trier et al 1967 Ahonen 1970) and dopamine (Chapter III) stimulate the secretion of the Paneth cells and vagotomy (Chapter III) and atropine inhibit it (Trier et al. 1967). The Paneth cell granules are able to take up catecholamines (Penttilä and Ahonen 1969 Ahonen and Penttilä 1969 1970 1971) cysteine and thryptophan (Metzel 1967) leucine (Trier et al. 1967) glucose and in some extent also arginine (Halhuber et al. 1972). Milk diet doubles the content of the Paneth cells of the mouse (Recknagel et al 1972). In the present study the effect of chemical sympathectomy on the Paneth cells was examined as there are no reports on the effects of sympathectomy on the Paneth cells available at present.

Material and methods

About 100 adult albino mice of both sexes were used. The animals were fasted one day before experiments and samples were taken from the mid-duodenum and midjejunum.

Sympathectomy was made by injecting 50 mg of 6-hydroxydopamine chloride (Hoffman La Roche) in saline solution intraperitoneally 2—14 days before experiments. Samples were taken from the mid-duodenum and mid-jejunum and stained with Thioflavine T and Best's carmine. Paneth cell granules were calculated as in Chapter III in 4 different animals and 6 adjacent sections and of total 600 Paneth cells in each group.

For fluorescence histochemical methods the Paneth cell granules were labelled in the sympathectomized as well as the control groups by injecting intraperitoneally dopamine chloride (Orion) 160 mg/kg intraperitoneally 1 hour after nialamide (Niamid[®] Pfizer) 500 mg/kg. The animals were killed 5 10 15 30 45 and 60 minutes 2 3 4 5 and 6 hours after the dopamine injection. Specimens were taken and treated for formaldehyde-induced fluorescence according to standard methods.

Results

The formaldehyde-induced fluorescence (FIF) of the adrenergic nerve fibers of the Intestinum totally disappeared after treatment with 6-hydroxydopamine. After such sympathectomy the Paneth cell granules were greater than those in the control group and the whole cell was filled with granules (Fig. 32).

The secretion speed of the Paneth cell granules was studied fluorescence histochemically with FIF after labelling the granules with dopamine, which is specifically taken up to these granules (Penttilä and Ahonen 1969, Ahonen and Penttilä 1971). After 6-hydroxydopamine injection the green fluorescence in the granules labelled with dopamine disappeared about 1 hour later than that in the control group (Table II).

Dopamine fluorescence accumulated in the Paneth cell granules at about the same rate in the 6-hydroxydopamine injected and in the control mice. Five minutes after the dopamine injection the whole Paneth cell was visible as non-fluorescent against the other strongly fluorescent cells (Fig. 33). Ten to fifteen minutes after the dopamine injection the fluorescent material began to accumulate in the Paneth cell granules in the Golgi region (Figs. 34–37) and the nuclear membrane also became fluorescent and a large fluorescent particle probably nucleolus appeared inside the nucleus (Fig. 34 and 35). At the same time, also weakly fluorescent granular cells appeared in the intestinal epithelium and the lamina propria (Fig. 34 and 36). These cells were granular and stained with eosin. They were possibly eosinophil leukocytes which some time later also appeared higher up in the crypts and intestinal villi (Fig. 38).

Two weeks after sympathectomy with 6-hydroxydopamine the crypt basement membrane began to deform and white material appeared beneath the basement membrane. At the same time the intestinal villi also began to deform. In the most deformed crypts there were no typical Paneth cells left (Fig. 39).

The effect of chemical sympathectomy was also studied by calculating the average number of granules per cell in each group: a total of 600 Paneth cells. It showed that sympathectomy increased the number of granules per cell from 12.1 ± 3.2 to 16.5 ± 4.1 in the duodenum and from 8.0 ± 1.8 to 17.0 ± 5.3 in the jejunum. The differences were significant ($p < 0.001$). Dopamine decreased the granule content significantly (Fig. 40 and 41). As to the jejunal Paneth cells the effect of dopamine was greater after sympathectomy indicating that jejunal Paneth cells became sensitive against dopamine by 6-hydroxydopamine treatment. Dopamine decreased the granule content from 17.0 ± 5.3 after sympathectomy to 10.8 ± 2.9 in the jejunum.

In the duodenum the decrease in the granule content caused by dopamine after sympathectomy was from 16.5 ± 4.1 to 13.7 ± 3.2 (Fig 40 and 41). All the differences were significant ($p < 0.001$). After sympathectomy the average number of granules per cell was about the same in the duodenal and in the jejunal Paneth cells ($0.1 > p > 0.05$) whereas the control group showed significantly higher ($p < 0.001$) number of granules per cell in the duodenum (Fig 40 and 41).

Discussion

The compound 6-hydroxydopamine produces a long lasting depletion of noradrenaline from peripheral sympathetically innervated tissues in various species. The denervation takes place through degeneration and possibly disappearance of the peripheral adrenergic nerves (Uretsky and Iversen 1969, Hökfelt et al. 1972). During this sympathectomy acetylcholine or choline acetyltransferase activities are not altered (Consolo et al. 1972).

According to the present study sympathectomy with 6-hydroxydopamine inhibited the secretion of the Paneth cells more than did vagotomy. In the present study both duodenal and jejunal Paneth cells had the same capacity to form and secrete granules although the number of granules in the jejunal Paneth cells was normally smaller. After sympathectomy the granules in the Paneth cells of the mouse were greater than in the mice of the control group after vagotomy which may mean that parasympathetic stimulus has greater effect on the granule synthesis as had been proposed also by Trær et al (1967). However the parasympathetic stimulus influences also the secretion process to some extent. Sympathetic stimulus does apparently not have as great an effect on the synthesis but it is needed for the normal basal secretion.

After chemical sympathectomy the jejunal Paneth cells became sensitive towards dopamine. This is in agreement with the observation that 6-hydroxydopamine produces supersensitivity to noradrenaline in perfused rat mesenterum (Finch and Leach 1970) and aorta of the rat (Shibata et al. 1972) and the sympathetic hyperinnervation produces hyposensitivity to noradrenaline (Kuzmicheva et al. 1969). Cyclic adenosine monophosphate has been shown to be a transmitter of the sympathetic stimulus in many organs (Takagi et al. 1971, Andersson 1972, Kaliner et al. 1972, Nakanishi and Takeda 1972, Smythies 1972) and 6-hydroxydopamine has been shown to produce an increased cyclic adenosine monophosphate response to noradrenaline (Palmer 1972). It can be therefore expected that cyclic adenosine monophosphate may serve as a modulator of adrenergic stimulation also in the Paneth cells of the mouse.

The observation that dopamine is also bound to a great particle inside the nucleus which may be nucleolus may have some connections with the discovery that noradrenaline stimulates polyamine synthesis (Caldarera et al. 1970)

The presence of adrenergic nerve fibers in close contact with the crypt basement membrane has been shown earlier (Costa and Gabella 1967 Gabella and Costa 1968 Silva et al. 1968)

The presence of adrenergic-cholinergic interactions has been proposed by many authors (Åberg and Eränkö 1967 Rauanheimo and Eränkö 1968 Modi et al. 1968 Tacca et al. 1970 Elfin 1971 a, b = Kosterlitz and Leeds 1972 Consolo et al. 1972 Kanerva and Hervonen 1972) If such interactions exist in the nerves that regulate the secretion of the Paneth cells it seems reasonable that cholinergic stimulus may be modulated through adrenergic receptors because the specific adrenergic peripheral denervation with 6-hydroxydopamine the nature of which has been widely studied (Thoenen and Tranzer 1968 Uretsky and Iversen 1969 Finch and Leach 1970 Goaling and Dixon 1972 Hückfelt et al. 1972) had greater inhibitory action on the Paneth cells of the mouse than vagotomy inhibiting the secretion of the Paneth cells most.

TABLE No II

Effect of sympathectomy on the secretion of the Paneth cells of the mouse

Sympathectomy was made by injecting 50 mg/kg 6-hydroxydopamine intraperitoneally 2-14 days earlier Nialamide was injected intraperitoneally 500 mg/kg and dopamine 160 mg/kg one hour later Thereafter the animals were killed at different intervals after dopamine injection. The control group received only nialamide and dopamine. The intensity of fluorescence in the Paneth cell granules was estimated visually and each column is a mean value of 3-4 experiments.

Explanation: — = no fluorescence in the Paneth cell granules,
+ = trace of fluorescence, ++ = weak fluorescence,
+++ = moderate fluorescence and +++++ = strong fluorescence

Time after dopamine	10 min	30 min	45 min	60 min	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs
SYMPATHECTOMY									
duodenum	+	++	++	++++	+++++	++	+	—	—
jejunum	+	++	++	++++	+++++	++++	++	+	—
CONTROL									
duodenum	+	++	++++	+++++	++	+	—	—	—
jejunum	+	++	++++	+++++	++++	++	+	—	—

V EFFECT OF DECARBOXYLASE INHIBITION ON THE L-DOPA UPTAKE BY THE PANETH CELLS OF THE MOUSE AND THE CRYPT BASEMENT MEMBRANE

Introduction

Paneth cells take up exogenous catecholamines in their special secretory granules and this uptake is intensified by monoamine oxidase inhibition and prevented by imipramine or cocaine (Penttilä and Ahonen 1969 Ahonen and Penttilä 1969 1970 1971) It has been proposed earlier that also Paneth cells belong to the APUD (Amine Precursor Uptake and Decarboxylation) cells (Ahonen and Penttilä 1971) In the present study the effect of decarboxylase inhibition on the L-dopa uptake was investigated by using the formaldehyde induced fluorescence (FIF) method for histochemical demonstration of catecholamines (Erilinkö 1967) So far as I know no such study has been carried out earlier and it would be of interest to know if there is decarboxylase activity in the Paneth cells of the mouse because it is one of the properties of the APUD cells

Material and methods

The material consisted of 40 albino mice of both sexes of an unknown strain used in the Department of Anatomy

The following drugs were used nialamide (Niamid[®] Pfizer) 500 mg/kg intraperitoneally 1—2 hours before L-dopa or dopamine injection, the decarboxylase inhibitor Ro 4602 (Hoffman La Roche) 40 mg/kg intraperitoneally 30 minutes before L-dopa injection L-dopa (Orion) 160 mg/kg intravenously and dopamine chloride (Orion) 160 mg/kg intravenously Both L-dopa and dopamine were injected 5 15 30 60 minutes, 2 4 and 5 hours before killing the animals All the drugs were dissolved in physiologic saline. To dissolve L-dopa a small amount of 1 N HCl was used

Results

It was observed in the present work that leukocytes penetrate into the crypt lumen between two Paneth cells at the bottom of the crypt (Fig. 42) The Paneth cell cytoplasm was non-fluorescent immediately after dopamine injection in contrast to the other epithelial cells which were diffusely

fluorescent (Fig 43 and 45) At the same time there was also some large fluorescent particles visible in the nuclear region of the Paneth cells (Fig 43) Ten minutes after dopamine injection the fluorescent material began to accumulate in the apical secretory granules of the Paneth cells of the mouse (Fig. 44) The number of fluorescent secretory granules increased soon thereafter

The crypt basement membrane was also strongly fluorescent soon after dopamine injection and remained fluorescent for 1.5 hours (Fig 43 44 and 45) After chemical sympathectomy by 6-hydroxydopamine the crypt basement membrane was loosened from the epithelial cell lining and both the basement membrane and the cell membrane of the Paneth cells became fluorescent (Fig. 45)

After decarboxylase inhibition with Ro 4602 and L-dopa injection the cytoplasm of the Paneth cells and the crypt basement membrane remained negative whereas the other epithelial cells received their strong green diffuse fluorescence (Fig 46) No accumulation of the fluorescent material in the secretory granules took place (Fig 46) as compared to controls (Fig 44) The only green fluorescent cells after Ro 4602 and L-dopa injection were some mast cells and small eosinophil leukocytes which had some big strongly fluorescent granules visible higher up in the crypts and villi and sometimes also in the cryptal lumen (Fig 47)

Discussion

The Paneth cell granules are able to take up L-dopa and dopamine (Penttilä and Ahonen 1969) According to the present work it was possible to prevent the appearance of the L-dopa induced fluorescence in the Paneth cell granules by decarboxylase inhibition with Ro 4602 This suggests that some decarboxylase activity exists in the Paneth cells and that L-dopa cannot be taken up by these cells unless it is first decarboxylated to dopamine. The presumed decarboxylase activity in the Paneth cells would further support the earlier proposal that Paneth cells belong to the APUD (Amine Precursor Uptake and Decarboxylation) series of cells (Ahonen and Penttilä 1971)

The lack of fluorescence of the crypt basement membrane after Ro 4602 and L-dopa injection may mean the presence of decarboxylase activity in the crypt basement membrane. If there is however no decarboxylase activity in the basement membrane, the only explanation is that dopamine is bound

to the basement membrane substance inside the Paneth cells and transferred thereafter to the site of the basement membrane with a rather fast turnover time. It has been shown that the epithelial basement membrane is universally secreted by adjacent epithelial cells (Pierce and Nakane 1972) which may favour the latter with the above possibilities but does not exclude the former.

The enzymes needed for the production of melanine from L-dopa or L-tyrosine have been found in the eosinophil cells and the mast cells (Okum et al 1970). The accumulation of L-dopa into these cells after decarboxylase inhibition observed in the present work suggests that the production of melanin is favoured after decarboxylase inhibition with Ro 4602 in the small intestine of the mouse. This may have some connections with the abnormal formation of melanin pigment which sometimes occurs in organs which normally have no pigments.

PART THREE

VI ORTHO PHTHALALDEHYDE INDUCED FLUORESCENCE IN THE PANETH CELLS OF THE MOUSE

Introduction

It is possible to demonstrate Paneth cells using formaldehyde-induced fluorescence because the cells take up dopamine and L-dopa into their secretory granules (Penttilä and Ahonen 1969, Ahonen and Penttilä 1969) and this uptake can be intensified by monoamine oxidase inhibition and blocked by imipramine and cocaine (Ahonen and Penttilä 1970, 1971).

The specificity of formaldehyde-induced fluorescence (FIF) first described by Erikkö in 1952 is well established (see Erikkö 1967). Ortho-phthalaldehyde induced fluorescence (OPTIF) has been shown to demonstrate histamine (Shore et al. 1959, Ehinger et al. 1968, Shelley et al. 1968, Shelley and Öhman 1968), histidine (Ehinger and Thunberg 1967), arginine, 5-hydroxyindoleamines, secretin, glucagon and vasoactive intestinal peptide (Håkanson et al. 1971, Polak et al. 1971, Håkanson et al. 1972). OPTIF visualizes also mast cells (Ehinger et al. 1968, Shelley et al. 1968, Shelley and Öhman 1968), pancreatic islets (Takaya 1970) and sweat ducts (Öhman and Shelley 1969). In the present work the localization of OPTIF (Ehinger et al. 1968, Enerbäck 1969, Takaya 1970) was studied in the intestinal cells by using a method in which epoxy resin sections were used to facilitate the localization of the reaction.

Material and methods

The material consisted of 21 adult albino mice of both sexes. The samples were taken from different parts of the Intestinum and treated for formaldehyde-induced fluorescence according to standard methods (see general material and methods)

The Epon sections were viewed in the fluorescence microscope for catecholamines and provided with the same filters as earlier. Thereafter the sections were moistened with a small amount of water to dissolve the FIF. One drop of 1 % o-phthalaldehyde in xylene was put on the slide and the OPTIF was examined with the fluorescence microscope using the same filters. If wanted, the same sections were stained later on with toluidine blue. The color of the FIF due to catecholamines was green and that of OPTIF yellowish. Some sections were stained without being moistened with water and it was observed that no OPTIF developed.

All the experimental animals except those of the normal control group were given 500 mg/kg nialamide (Niamid[®] Pfizer) intraperitoneally 3—5 hours before and dopamine chloride (Orion) 160 mg/kg 15 and 30 minutes 1 2 3 and 4 hours before being killed.

Results

After abolishing the FIF (Fig 48 and 49) from a freeze-dried Epon section by a small amount of water it was possible to develop OPTIF in the same section while observing it in the fluorescence microscope. Water abolished the FIF rapidly. On the other hand, it was not possible to obtain OPTIF without moistening the section with a small amount of water. Paneth cells were identified after dopamine injection by their green fluorescent granules and the enterochromaffin cells by their yellow FIF. Without dopamine treatment the Paneth cells showed no FIF in their granules.

The mast cells in the lamina propria were visible with both FIF and OPTIF (Fig 50). The secretory granules of the Paneth cells of the mouse were distinctly fluorescent with OPTIF also without dopamine pretreatment (Fig. 50 51 52 and 53). More granules per cell were visible with OPTIF as compared with FIF indicating that not all the Paneth cell granules take up dopamine after a single injection (Fig. 52).

Small granular cells appeared the cryptal lumen exhibiting a strong green FIF in their granules about 1 hour after dopamine injection. Later these granular cells were also visible higher up in the crypts and the lamina propria. These granular cells which stained with eosin and exhibited also OPTIF were morphologically different from the mast cells (Fig 50 51). The

number of these OPTIF positive cells increased after dopamine injection

In the colonic Paneth cells the OPTIF was more diffuse and not so distinctly granular as in the small intestine. The nerve fibers were non-fluorescent after the water and showed no OPTIF indicating that this was not due to catecholamines. The crypt basement membrane also exhibited a clear OPTIF (Fig 53)

Discussion

It was shown in the present study that OPTIF was demonstrable in the Paneth cell granules in the enterochromaffin cells and in the mast cells. The Paneth cell granules are known to contain arginine and histidine (Taylor and Flax 1964 Merzel 1967). It has been shown that both arginine and histidine have "protected linkages" in the Paneth cell granules while no arginine and histidine can be found in the pancreatic acinar cells (Merzel 1967). This may mean that histidine and arginine are bound to peptides.

Glucagon and secretin which also contain NH_2 -terminal histidine also exhibit OPTIF whereas inulin the thyroid-stimulating hormone and thyrotropin releasing hormone which are devoid of histidine residue do not show OPTIF (Håkanson et al 1971 Håkanson et al 1972). The pancreatic islets show OPTIF and this is probably due to glucagon (Takaya 1970 Håkanson 1971). The OPTIF of the mast cells is probably not due to histamine (Ehinger et al 1968 Shelley et al 1968 Shelley and Öhman 1968).

The OPTIF in the Paneth cell granules may be due to arginine, to histidine or to both of them. The Paneth cells are the greatest cell population in the intestine exhibiting OPTIF. Thus they are probable site of the vasoactive intestinal peptide which also is OPTIF positive (Håkanson et al 1972). It has been proposed earlier (Ahonen and Penttilä 1971) that Paneth cells belong to the polypeptide producing APUD (Amine Precursor Uptake and Decarboxylation) cell system of Pearse (1969) being able to take up catecholamines and their precursors. Moreover it has been shown that there is decarboxylase activity in the Paneth cells (Chapter V). If the vasoactive intestinal peptide (Håkanson et al 1972) is the polypeptide secreted by the Paneth cells it would be understandable that these cells belong to the APUD cell system which are polypeptide producing cells (Pearse 1969).

In the present study the enterochromaffin cells and the Paneth cells were identified by their FIF and no attempts were made to identify the rare enteroglucan cells which also have been shown to exhibit OPTIF (Unger et al 1966 Polak et al 1971).

VII GENERAL DISCUSSION

Uptake mechanism and secretion

The uptake of dopamine and L-dopa by the Paneth cells of the mouse has been described first by Penttilä and Ahonen (1969). This uptake can be intensified by monoamine oxidase inhibitors nialamide and pargyline hydrochloride, and prevented by cocaine and imipramine (Ahonen and Penttilä 1970, 1971) which are inhibitors of the amine pump at the cell membrane level (Musbohl 1961, Berti and Shore 1967, Reiffenstein 1968, Ross et al. 1971). Reserpine, promazine, amphetamine, amitriptyline and nortriptyline have no effect on this uptake (Ahonen and Penttilä 1971). Chlorpromazine is known to have a central anticholinergic activity (Conasroe and White 1972) and it liberates adrenaline from the adrenal medulla (Vapaatalo et al. 1969) but has no effect on the dopamine uptake of the Paneth cells of the mouse (Ahonen and Penttilä 1971).

Guanethidine is known to induce chemical sympathectomy (Eränkö and Eränkö 1971, Heath et al. 1972) and it releases catecholamines from the adrenal medulla (Jaanus et al. 1968). It has no effect on the dopamine uptake of the Paneth cells of the mouse (Ahonen and Penttilä 1970, 1971). Neither had 6-hydroxydopamine an inhibitory effect on the dopamine uptake but instead it inhibited the secretion of the Paneth cells (Chapter IV) more than vagotomy (Chapter III) which in turn, had an inhibitory effect also on the granule synthesis (Trier et al. 1967). Possible effects of guanethidine on the Paneth cell secretion have not been studied. Noradrenaline is shown to decrease the mesenteric blood flow whereas acetylcholine increases it (Shehadeh et al. 1969). Total bilateral vagotomy decreases also the mucosal blood flow (Padula et al. 1968). Both sympathectomy (Chapter IV) and vagotomy (Chapter III) have inhibitory effect on the secretion of the Paneth cells. This effect cannot therefore be explained merely by a decreased mucosal blood flow which may have some effect on the gastric secretion (Jacobson 1969) but it may have some postvagotomy influences.

Alpha-methyl-Dopa, noradrenaline, alpha-methyl-noradrenaline, and adrenaline are also taken up to the secretory granules of the Paneth cells of the mouse but in a much smaller degree than L-dopa or dopamine, and it is not even possible to detect the uptake of alpha-methyl-noradrenaline and adrenaline without monoamine oxidase inhibition (Ahonen and Penttilä 1971). Tyrosine, alpha-methyl-tyrosine, 5-hydroxytryptamine and 5-hydroxytryptophan are not taken up to the Paneth cell granules of the mouse in a detectable degree (Ahonen and Penttilä 1971). The uptake of labelled tryptophan

and cysteine is similar in the Paneth cells and the pancreatic acinar cells (Merzel 1967)

Protein synthesis and amino acid secretion

Five minutes after dopamine injection the strong green fluorescence was seen throughout the whole intestinal mucosa except for the Paneth cells (Chapter V) Five minutes later fluorescent dopamine began to accumulate in the apical secretory granules of the Paneth cells of the mouse At that time the nucleolus of the Paneth cells was occasionally also slightly fluorescent (Chapter IV) The number of the fluorescent granules reached its maximum from 30 minutes to 2 hours after dopamine injection (Chapter III) Fluorescent granules were secreted into the crypt lumen from 2 to 4 hours after injection (Chapter III) Dopamine stimulated the secretion of the Paneth cells significantly (Chapter III and IV) so that the real basal Paneth cell secretion takes place more slowly than can be seen by dopamine labelling This secretion speed is however rather similar to that received by labelling the Paneth cell granules with leucine (Trier et al. 1967) This Paneth cell granule synthesis and secretion is also almost identical to the pancreatic acinar cell secretion (Alm et al. 1971 Krumer and Poort 1972 Palade 1972 Wormesley 1972)

According to the present study the Paneth cell granules may contain vasoactive intestinal peptide which has been shown to be secreted by the intestinal mucosa (Håkanson et al. 1972) This possible polypeptide secretion fits well in the assumption that the Paneth cells belong to the APUD cell series (Ahonen and Penttilä 1971) A striking feature of these APUD cells is that most but not all of them, are in glands or tissues which during organogenesis have developed from the primitive gut as for instance stomach pancreas lungs pituitary thyroid and larynx (Langman 1963 Pearse 1968 Rost et al. 1969 Pearse and Polak 1971 Hage 1973) It has been proposed that APUD cells are derived from the neural crest (Pearse and Polak 1971) According to the present and earlier observations (Ahonen and Penttilä 1971 Chapter V and VI) the Paneth cells fulfill all the other criteria of the APUD cells with the exception that their origin is still unknown.

Common origin of enterochromaffin-like cells and Paneth cells

In the glandular part of stomach there are enterochromaffin-like (EC-like) cells which are argyrophil but not argentaffin and which take up L-

dopa like the Paneth cells and like them show ortho-phthalaldehyde induced fluorescence (OPTIF) (Aures and Håkanson 1968 Håkanson et al. 1969 Håkanson et al. 1969 Tjälve 1971) According to the present study the Paneth cells exhibit OPTIF (Chapter VI) probably due to histidine or arginine or both. These two are known to exist in the Paneth cell granules (Merial 1967) They may be components of the vasoactive intestinal peptide which can also be visualized with the OPTIF method (Håkanson et al. 1972) and as the Paneth cells are the only big OPTIF positive cell group in the Intestinum (Chapter VI) the only possibility is that vasoactive intestinal peptide is secreted by the Paneth cells During a severe atrophic gastritis Paneth cells regularly appear in the mucosa of the human stomach (for ref see Otto and Fett 1972) which normally do not contain these cells.

The FIF of both EC-like cells (Håkanson 1970) and Paneth cells after L-dopa depends on intact decarboxylase activity (Chapter V) It has been proposed that the ability to take up amines or their precursors is a common feature of a wide embryologically related cell group (Pearse 1969) thus it is proposed according to the results of the present work (Chapter V and VI) that EC-like cells and Paneth cells may be differentiated from a common ancestor cell type which resembles more the Paneth cells During a severe atrophic gastritis these EC-like cells can possibly be converted into Paneth cells. According to this the Paneth cells may be the least differentiated secretory cell type in the gastrointestinal canal.

Colitis ulcerosa and Paneth cells

Lysozyme activity has been shown in the granule fraction of the Paneth cells (Deckx et al. 1967 Ghooos and Ventrappen 1971) During ulcerative colitis small intestinal Paneth cells resembling cells appear into the human colon (Lauren 1961 Toner 1968 Otto and Fett 1972) It has been shown earlier that secretion of the Paneth cells is accelerated by sympathetic and parasympathetic stimulation (Chapter III and IV) and that parasympathetic stimulation has some influence also on the granule synthesis (Trier et al. 1967) and that both sympathectomy and parasympathectomy inhibit the secretion (Chapter III and IV) It has been proposed that etiology of the ulcerative colitis may be sacral parasympathetic hyperactivity (Boyd 1961) It has also been shown that colonic motility of the ulcerative colitis patients is decreased (Bloom et al. 1968) The decreased colonic motility may also reflect the increased sympathetic tonus in the colon which is known to have an inhibitory effect on the taenia coli (Lum et al. 1966

Bülbring et al. 1969 Ohkawa et al. 1972) Ulcerative colitis patients secrete abnormally great amounts of lysozyme into the feces and ulcerations are often in the taenial region (Boyd 1961) where the number of Paneth cells per surface area of mucosa is greatest (Chapter II) Thus the increased lysozyme secretion during ulcerative colitis may be a reflection of increased sympathetic and/or parasympathetic stimulation on the Paneth cells which in turn may induce during long time dedifferentation of the colonic Paneth cells into the small intestinal stage.

Crypt basement membrane

The crypt basement membrane shows intense FIF after injection of a large dose of dopamine and this fluorescence is similar to that observed in the secretory granules of the Paneth cells (Chapter V) The other epithelial cells in the intestinal crypts have not such dopamine binding ability (Ahonen and Penttilä 1971) After decarboxylase enzyme inhibition and L-dopa injection the crypt basement membrane is also non-fluorescent similarly to the Paneth cell granules (Chapter V) The crypt basement membrane or its dopamine-binding component may be secreted by the Paneth cells in the same way as the epithelial cells (Pierce and Nakane 1972) The basement membrane is possibly composed of a single glycoprotein without hydroxyproline and hydroxylysine (Johnson and Starcher 1972) The core of the Paneth cells granule is also muco or glycoprotein (Schman and Liebelt 1961 1962 a b Taylor and Flaas 1964)

According to the present work the cell membrane of the Paneth cells seems to be different from the other usual epithelial cells of the intestine at least functionally because it prevents the passive diffusion of dopamine into the cell (Chapter III IV and V)

Crypt morphology and Paneth cells

It has been shown recently that milk diet increases the number of Paneth cells (Recknagel et al. 1972) Diet lactation and intestinal bacterial flora inhibit the cell migration in the intestinal villi (Koldovsky et al. 1966) and influence also the mucous membrane morphology (Pfeiffer 1968 Herbst and Sunshine 1969 Lochry and Creamer 1969 a, b) Artificial feeding produces precocious development and maturation of the intestinal crypts (Herbst and Sunshine 1969) When the morphology of the ileum and the

jejunum begins to deform the first pathologic step is the formation of intervillous ridges, after which the crudal small intestine begins to resemble the normal duodenum (Loehry and Creamer 1969 a, b) This "duodenalisation" is the first pathologic step during lactation, intestinal bacterial infection, recovery after methotrexate treatment or during coeliac disease (Loehry and Creamer 1969 a, b) It is interesting to notice that the number of Paneth cells is significantly smaller in the duodenum than in the crudal small intestine and the first pathologic alterations during malabsorption can be detected in the duodenum (Toner 1968) It has been proposed that crypt morphology remains normal as long as there are Paneth cells left in the intestine but that morphology is disturbed if the Paneth cells are lacking (Creamer 1968) In the present work the crypt basement and villous morphology was disturbed if the secretion of the Paneth cells was inhibited by chemical sympathectomy with 6-hydroxydopamine (Chapter IV) Vagotomy had not so great inhibitory effect on the secretion of the Paneth cells and was not able to disturb the crypt morphology Paneth cells appeared in the small intestine of the mouse at the time when the crypt began to develop (Chapter I) which may support the above hypothesis together with the earlier observations

The effect of the Paneth cells on the crypt morphology can possibly be explained simply by the different mitotic frequencies between the Paneth cells and the other epithelial cells in the crypt. It has been shown that Paneth cells never divide (Deschner 1967 Cheng et al. 1969 Troughton and Trier 1969) whereas the other epithelial cells round them divide rapidly resulting in the Paneth cells remaining between them like at the bottom of a well

MAIN NEW RESULTS AND CONCLUSIONS

- 1 Dopamine stimulated secretion of the Paneth cells
- 2 Vagotomy inhibited secretion of the Paneth cells
- 3 Chemical sympathectomy with 6-hydroxydopamine almost totally prevented secretion of the Paneth cells and caused deformation of the intestinal crypt basement membrane, crypts and villi.
- 4 Duodenal Paneth cells had significantly more granules than the jejunal ones but both duodenal and jejunal Paneth cells had the same capacity to form and secrete granules.
- 5 The effect of sympathectomy and vagotomy was greater on the jejunal than the duodenal Paneth cells.

- 6 Crypt development began at the same time as the appearance of the Paneth cells
- 7 Paneth cells received their dopamine uptake ability at the age of three weeks which possibly means the beginning of the polypeptide or amino acid secretion.
- 8 Paneth cells reached their normal adult number and dopamine uptake ability at the age of about 4 weeks
- 9 The cells at the bottom of the colonic crypts were able to take up dopamine. They were possibly related to small intestinal Paneth cells
- 10 The crypt basement membrane took up L-dopa but did not show formaldehydeinduced fluorescence after decarboxylase inhibition and L-dopa injection
- 11 The crypt basement membrane took up dopamine. It may be formed and secreted by the Paneth cells
- 12 There was ortho-phthalaldehyde induced fluorescence (OPTIF) in the Paneth cell granules and the crypt basement membrane.
- 13 Cholinergic and adrenergic nerve fibers were in close relationship with the crypt basement membrane but they never penetrated it.
- 14 Paneth cells were possibly related with the EC-like cells of the stomach.

FIGURES

Figures 1—4 are Thioflavine T fluorescence photographs of sections of the developing mouse fixed with the buffered formaldehyde.

Fig. 1. The cross section of the of the duodenum of a two-weeks-old mouse where fluorescent Paneth cell granules can be seen at the bottom of one crypt (arrow) No fluorescent goblet cells are visible. 250×

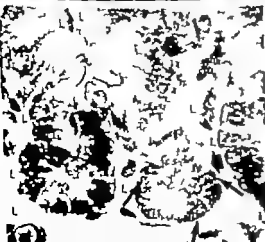
Fig. 2. The colon of the two-weeks-old mouse where the fluorescent round goblet cells are visible, and the basal epithelial cells at the bottom of the colonic crypts (colonic Paneth cells) are also fluorescent and not well distinguished from the goblet cells. 250×

Fig. 3. The jejunum of the four-weeks-old mouse where fluorescent Paneth cells are well visible at the bottom of the crypts. No fluorescent cells can be seen higher up in the crypts or the villi. The number of Paneth cells has reached its normal adult level. 250×

Fig. 4. The ileum of a three-weeks-old mouse where some fluorescent granular Paneth cells are visible. Also some goblet cells can be seen. 250×

Fig. 5. A section of the duodenum of a two-weeks-old mouse fixed with formaldehyde and stained with Best's carmalum 2 hours after nialamide and 30 minutes after dopamine injection. The long arrows indicate three Paneth cells, the granules of which are still pale stained. One eosinophil cell can be seen higher up in the crypt (short arrow) White cytoplasmic round nuclear cells in the lamina propria (marked L) possibly represent lymphoid cells. 400×

Fig. 6. A freeze-dried section of the duodenum of the three-weeks-old mouse embedded in paraffin. The section is taken 2 hours after nialamide and 30 minutes after dopamine injection and treated for formaldehyde induced fluorescence according to the standard method. The Paneth cells at the bottom of the crypts are already capable of taking up dopamine. 250×

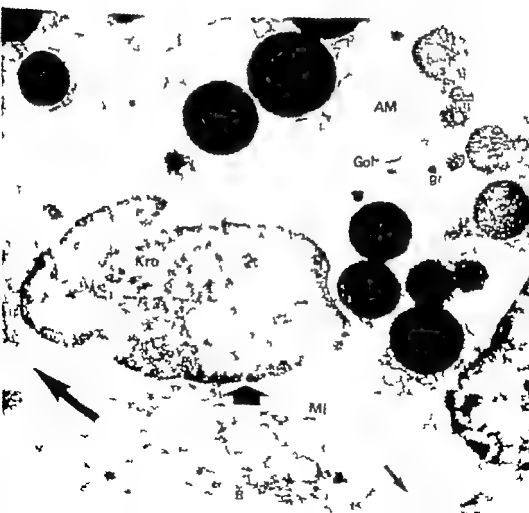


Figures 7—9 are electron microscopic photographs of the sections of the duodenum of the developing mouse fixed with glutaraldehyde-osmium tetroxide and poststained with lead citrate and uranyl acetate.

Fig. 7 shows the apical part of the duodenal Paneth cell of the mouse with a well developed Golgi Complex (Gol) and a rich rough endoplasmic reticulum (RER). The specific secretory granules have a large halo (SGr) and 5 small electron dense bodies (grs) are visible in the Golgi region. 10,000×

Fig. 8. The basal part of the same cell as in Fig. 7. The cell has a prominent nucleolus (Kro) which is electron dense with one projection downwards and it seems to be continuous with the nuclear envelope on the left side. The cell has numerous free ribosomes in its cytoplasm (rib) and the mitochondria are big and round to oval shaped (mi). The rough endoplasmic reticulum in the upper part of the figure is marked with RER. This Paneth cell is very long and narrow projecting from the basement membrane (BM) up into the cryptal lumen. 10,000×

Fig. 9. An electron microscopic photograph of the duodenum of the four-weeks-old mouse where the nucleolus (Kro) is also prominent. In the nuclear membrane there are some pores (short thick arrow) visible. The mitochondria are big and of different shape (small arrow). The rich rough endoplasmic reticulum has many sac-like dilations (long arrow). Typical apical electron dense secretory granules or without a halo. Some small electron dense bodies (gr) are visible in the Golgi region (Gol). In the middle of the Golgi apparatus there is an area filled with amorphous material (AM). 12,000×



Figures 10—15 are 7 μ thick sections of the colon of the adult mouse fixed with formaldehyde buffered with phosphate.

Fig. 10 shows the section of the colon of the mouse stained with hematoxylin-eosin where it is difficult to identify the colonic epithelial cells at the bottom of the crypt from the goblet cells. 250 \times

Fig. 11 Section of the colon of the mouse stained with iron hematoxylin where it is possible to differentiate the goblet cells from the epithelial cells at the bottom of the crypts, but not very accurately 400 \times

Fig. 12. A fluorescence photograph of the colonic section of the mouse stained with Thioflavine T. This staining method makes it possible to differentiate the strongly fluorescent goblet cells from the basally not so strongly fluorescent epithelial cells of the crypt. 250 \times

Fig. 13. A Thioflavine T fluorescence photograph of the colon of the mouse near the caecum coli with more basally located epithelial cells per surface area of mucosa than in the other regions of the colon. These basal epithelial cells are morphologically different from the small intestinal Paneth cells. 250 \times

Fig. 14. A Thioflavine T fluorescence photograph of the colon of the mouse where the short arrow indicates a colonic basal epithelial cell or colonic Paneth cell with the apical secretory granules which are well visible. 250 \times

Fig. 15. A thioflavine T fluorescence photograph of the normal colon of the mouse where the colonic Paneth cells change into goblet cells in the upper part of the crypt. 250 \times

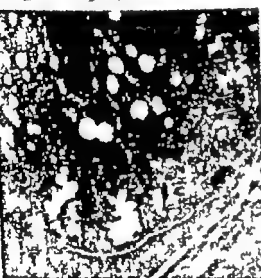


Fig. 16. A freeze dried Epon section of the normal colon of the mouse stained with toluidine blue 2 hours after nialamide and 30 minutes after dopamine injection. The short arrow indicates basally located epithelial cells in the crypt or so called colonic Paneth cells which can bear be identified by their prominent nucleolus and pale, slightly granular apical cytoplasm. Higher up in the crypt there are many dark stained, distinctly granular cells which seem not to be the usual goblet cells. The number of these granular cells is greater after dopamine injection than in the control group. 400X

Figures 17—20. Formaldehyde-induced fluorescence (FIF) photographs of the freeze-dried Epon sections 2 hours after nialamide and 30 min. after dopamine injection

Fig. 17. Colonic section of the four-weeks-old mouse where green dopamine fluorescence is located in the apical secretory granules of the colonic Paneth cells. Normally there is no catecholamine fluorescence in these cells. In the upper part of the figure there is no catecholamine fluorescence in these cells. In the upper part of the figure to 5HT 230X

Fig. 18. Colonic section of the adult mouse where green fluorescent granules are distinctly visible. 400X

Fig. 19. Fluorescent granules in the apical part of the colonic Paneth cells. 900X

Fig. 20. Green fluorescence in the small granules in the apical part of the cells against the dark cytoplasm. 400X



Fig. 21 An electron microscopic picture of the colon of the adult mouse. Basal epithelial cells in the crypt have paler secretion granules (SR) than the small intestinal Paneth cells but have well developed Golgi apparatus (G) and prominent nucleolus (NR). The arrows indicate the other granule type which is smaller and darker in the picture. In the cryptal lumen there are some coliformic bacteria (EC) amongst the secreted material. No real junctions seem to exist between these epithelial cells. 7,500X

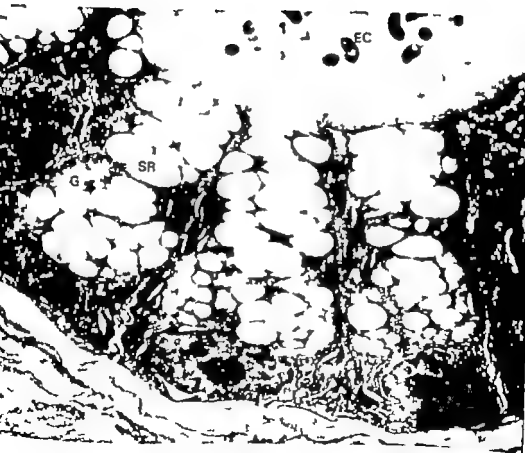


Fig. 22. An oil immersion light microscopic photograph of the freeze-dried Epon section, stained with toluidine blue, of the jejunum of the mouse 2 hours after nialamide (500 mg/kg) and 30 minutes after dopamine (160 mg/kg) injection. The section was kept for 2 hours in 60 C 0.1 N HCl solution before staining. The Paneth cell granules show a weak masked metachromasia. Fig. shows an oblique cross section near the crypt bottom. 900×

Fig. 23. An oil immersion formaldehyde induced fluorescence (FIF) photograph of the freeze-dried Epon section from the jejunum of the mouse 2 hours after nialamide and 10 minutes after dopamine injection. The other epithelial cells and the lamina propria are strongly fluorescent. The cytoplasm of the Paneth cells are dark, and in the apical part of the cells there are some Paneth cell granules already visible. 900×

Fig. 24. An oil immersion Thioflavine T fluorescence photograph of the section of the jejunum of the adult mouse fixed with formaline. The Paneth cell granules are strongly fluorescent and the basement membrane is also visible. 900×

Fig. 25. A FIF photograph of the ileum of the adult mouse 2 hours after nialamide and 30 minutes after dopamine injection. Fluorescent granular Paneth cells are visible in every crypt. The basement membrane is also fluorescent. 250×

Fig. 26. An oil immersion light microscopic photograph of the non-specific cholinesterase reaction in duodenum of the adult control mouse. There is positive reaction in a nerve fiber below the crypt basement membrane. The arrow indicates vacuoles near the Paneth cell. The Paneth cell granules are also positive. 1,000×

Fig. 27. An oil immersion photograph of acetylcholinesterase reaction in the duodenum of the adult control mouse. The stained granular nerve fiber (long arrow) is in close contact with the crypt basement membrane (short arrows) in the region of the Paneth cells. 1,200×



Fig. 28. Acetylcholinesterase reaction in the jejunum of the adult control mouse. Acetylcholinesterase positive nerve fibers are numerous in the muscle layer and one stained granular nerve fiber (arrow) is in close contact with the crypt basement membrane at the site of the Paneth cell. 250X

Fig. 29. An oil immersion photograph showing the section of the jejunum of the vagotomized adult mouse fixed with formaline and stained with Best's carmine. The figure shows the section through the long axis of the crypts. Seven big Paneth cells are filled with the typical secretory granules with a big halo. One lymphoid cell is near the basement membrane (arrow) outside it. 900X

Fig. 30. The number of Paneth cell granules per cell amounts to a total of 600 Paneth cells in the mid-duodenum and mid-jejunum 1 hour and 30 minutes after neisamide (500 mg/kg) and 30 minutes after dopamine (160 mg/kg) injection. The group treated with dopamine differs significantly from the control group ($p < 0.001$). The granules were calculated in the vagotomized group 24 hours after vagotomy and that, too, differs significantly from the control group. According to these calculations vagotomy inhibited and dopamine stimulated the secretion significantly.

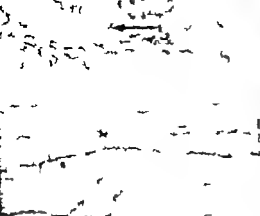


Fig 30

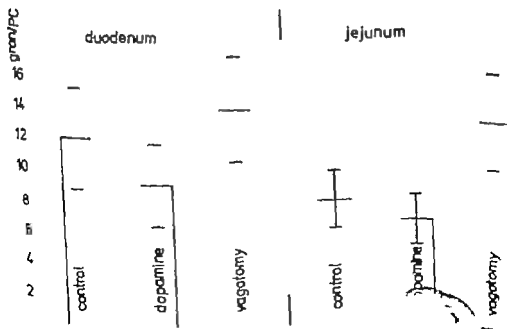


Fig. 31 The distribution of the Paneth cells of the mouse are given as a function of their number of granules. The same experiment groups are presented as in Fig. 30. The dotted line marks the average number of granules per cell. The dopamine and the vagotomized group both differ significantly from the control group.

Fig. 32. An oil immersion photograph showing the section of the jejunum of the adult mouse fixed with formaldehyde and stained with Best's carmine 2 days after 6-hydroxydopamine injection. The white cytoplasmic cells (L) are lymphoid cells. The Paneth cells at the bottom of the crypt are filled with big granules. 900×

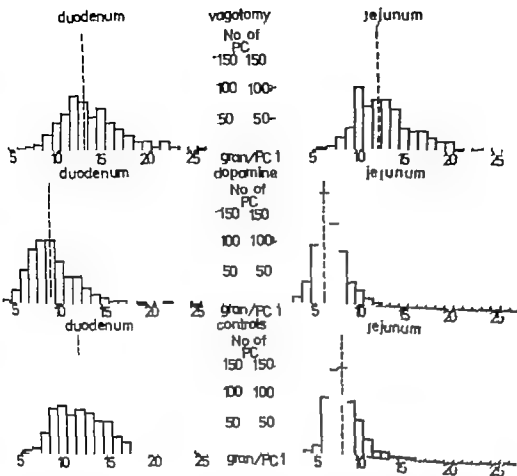


Fig. 32

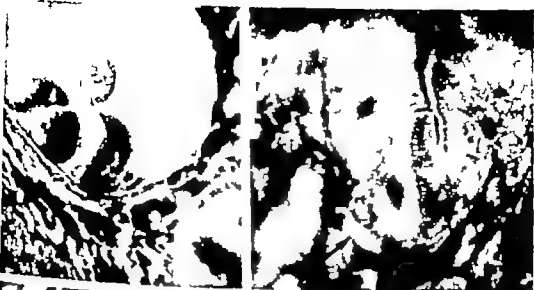


Fig. 34

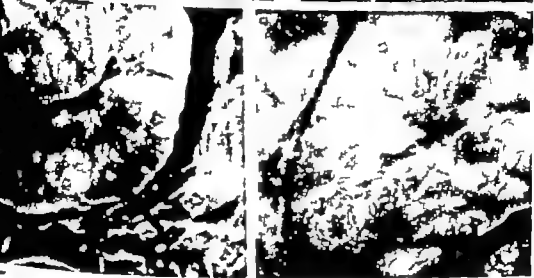


Fig. 36



Figures 33—38 FIF photographs of freeze-dried sections of the jejunum of the mouse embedded in Epon. The samples were taken 2 hours after nialamide (500 mg/kg) and 5 min. (Fig. 33) 15 min. (Fig. 34 35 and 36) and 30 min. (Fig. 37 and 38) after dopamine injection (160 mg/kg). The Epon sections in Figs. 33—36 are 2 μ thick and in Figs. 37 and 38 5 μ thick. All the mice in the Figs. 33—36 and 38 were given 6-lydivaydopamine (50 mg/kg) intraperitoneally 2 days earlier.

In Fig. 33 the cytoplasm of the Paneth cells is dark (arrow) against the other strongly fluorescent epithelial cells. 900 \times

In Fig. 34 a short arrow indicates a migrating fluorescent cell between the epithelial cells. Similar fluorescent granular cells can also be seen in the lamina propria (medium sized arrow). These moving granular cells are leukocytes, probably eosinophil leukocytes. Two long arrows indicate Paneth cells with dark stained cytoplasm and a slightly fluorescent nuclear membrane. Inside the nucleus there are slightly fluorescent particles visible which are probably nucleoli. 900 \times

In Fig. 35 an arrow indicates a Paneth cell with fluorescent granular material in its Golgi region. The nuclear membrane is slightly fluorescent and inside the nucleus there is one great fluorescent particle visible, probably nucleolus. 900 \times

In Fig. 36 an asterisk indicates a fluorescent cell, probably an eosinophil leukocyte, in the epithelium of the jejunum of the mouse. Outside the fluorescent cell membrane of this eosinophil cell there is fluorescent granular material in the Golgi region of the Paneth cell. 900 \times

In Fig. 37 two dark cytoplasmic Paneth cells are distinctly visible with their fluorescent apical cytoplasmic granules against the green fluorescent background. 900 \times

In Fig. 38 the crypts of Lieberkühn are cross sectioned at their mid-part. Strongly fluorescent cells are visible in the lamina propria outside the crypt basement membrane (arrow) 400 \times



Fig. 39

Fig. 39. An oil immersion photograph showing the section of the duodenum of the mouse fixed with formaldehyde and stained with Best's carmine two weeks after 6-hydroxydopamine injection (50 mg/kg). Three long arrows indicate the crypts with some Paneth cells at their bottom. Smallest arrow indicates the basement membrane of the crypts which is deformed and loosened from the epithelial cells of the crypts. In these deformed crypts there are no Paneth cells visible. The big arrows indicate the slightly deformed villi of the duodenum of the mouse. There are often great accumulations of white stained material (asterisk) below the deformed basement membrane. 250X

Fig. 42. An electron microscopic photograph of the jejunum of the mouse 1 week after sympathectomy. The mouse received nielamide 2 hours and dopamine 30 minutes before being killed. The microvilli of the Paneth cell are visible at the upper part of the figure. Large secretory granules with an irregular halo are visible. Between two Paneth cells there is one long process probably belonging to a leukocyte that penetrates into the crypt through the intercellular space. The process is full of big mitochondria and round grey granules. 15,000X

Figures 43—47 are formalin induced fluorescence photographs (FIF) of the jejunum of the mouse 2 hours after dopamine injection.

Fig. 43. The situation 10 minutes after dopamine injection. The cytoplasm of the epithelial cells excels begins to accumulate in Paneth cell (arrow). The propels are strongly fluorescent. 900X

Fig. 44. The situation 30 minutes after dopamine injection. The basement membrane is strongly fluorescent. On the left there is an empty enterochromaffin cell. Three Paneth cells are at the bottom of the crypt. Between the enterochromaffin and one cell, the cytoplasm of which is diffusely fluorescent and contains granules in its cytoplasm. 900X

Fig. 45. The situation 10 minutes after dopamine injection. The mouse was given 6-hydroxydopamine 1 week earlier. Five dark cytoplasmic Paneth cells are visible and dopamine begins to accumulate in the secretory granules. Between the two basal Paneth cells there is one strongly fluorescent cell. The space between the two Paneth cells is fluorescent and may belong to leukocytes that penetrate into the crypt lumen between the Paneth cells. A possibly similar process is visible by electron microscopy in Fig. 42. The basement membrane is strongly fluorescent (star) and is loosened from the also fluorescent cell membrane of the Paneth cell. 900X

Fig. 46. The situation 3 hours after Ro 4602 and 2 hours and 30 minutes after L-dopa injection. Three Paneth cells at the bottom of the crypt are totally negative. There is one partly emptied enterochromaffin cell (EC) between the Paneth cells. At the site of the Paneth cells and the enterochromaffin cell the basement membrane is negative. This negatively stained basement membrane makes a distinct contrast to the membranes in Figs. 43, 44 and 45. In those experiments decarboxylase inhibitor was not used. 1,000X

Fig. 47. The same experiment as in Fig. 46 but the picture is taken near the tip of the villus. One green fluorescent mast cell (MC) and three green fluorescent eosinophil cells (E) are visible. The eosinophil cells have great fluorescent granules in their cytoplasm. In the epithelial lining there are some small weakly fluorescent granules visible. 900X

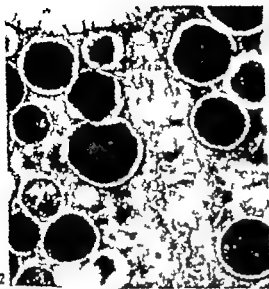


Fig. 42



Fig. 43



Fig. 44



Fig. 45



Fig. 46

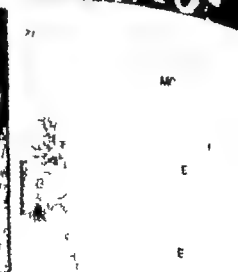


Fig. 49

Fig. 51

Fig. 48. FIF in the jejunum of the adult mouse. The mouse received nalamide (500 mg/kg) 3 hours and dopamine (160 mg/kg) 30 minutes before the killing. Fluorescent granules of the Paneth cells are visible. Green fluorescence is seen also in the basement membrane of the crypt. 200X

Fig. 49 FIF in the colon of the four weeks-old mouse. Drugs were given as in Fig. 48. Green fluorescent Paneth cell granules are visible against the dark cytoplasm. 250X

Fig. 50. OPTIF in the jejunum of the mouse. Drugs were given as in Fig. 48. The Epon section was moistened with water and one drop of 1% OPT in xylene was put on the slide. Fluorescence appeared in 10 minutes in the Paneth cells (P) and also in some other granular cells in the crypt lumen. One fluorescent cell in the lamina propria is a mast cell (arrow) and there is an enterochromaffin cell in the adjacent crypt that is also fluorescent with OPT (asterisk). The nerve fibers are non-fluorescent after moistening with water. 250X

Fig. 51. An OPTIF photograph of the ileum of the mouse treated as in Fig. 48. Nalamide was given 3 hours and dopamine 1 hour before killing. The Paneth cell (P) granules are distinctly fluorescent. More eosinophil fluorescent cells and higher up in the crypt as compared with Fig. 50 are visible. 250X



Fig. 48



Fig. 49



Fig. 50



Fig. 51

Fig. 52. OPTIF in the jejunum of the mouse. Nialamide was given 3 hours and dopamine 45 minutes before killing. More Paneth cell granules are visible as compared with FIF indicating that not all the granules become visible with FIF after a single dopamine injection. 250x

Fig. 53. OPTIF in the jejunum of the adult mouse. The mouse fasted two days after which it was allowed to eat 5 hours standard food, received nialamide 4 hours and dopamine 3 hours and 30 minutes before killing. Paneth cell granules are visible with OPTIF. Numerous cells in the lamina propria and submucosa are visible with OPTIF. The basement membrane is also fluorescent (arrow). 250x

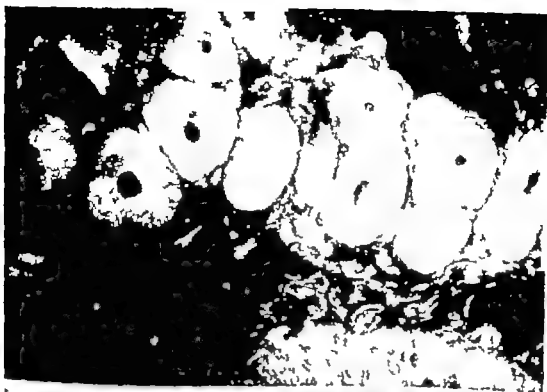


Fig. 52



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OXYGEN TRANSPORT
DURING EXERCISE
IN HUMAN SUBJECTS

by

Lars Hermansen

OSLO 1979

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From the Institute of Work Physiology, Oslo, Norway

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The present monograph is based on investigations carried out at the Institute of Work Physiology Oslo Norway the Department of Physiology Gymnastik och idrottshögskolan, Stockholm, Sweden and the Institute of Hygiene University of Oslo Oslo Norway Except for some unpublished results, this monograph includes results which have previously been reported in the following publications

- I HERMANSEN LARS and BENGT SALTIN Oxygen uptake during maximal treadmill and bicycle exercise.
J.appl.Physiol. 1969 26. 31-37
- II HERMANSEN LARS BJÖRN EKBLOM and BENGT SALTIN Cardiac output during submaximal and maximal treadmill and bicycle exercise.
J.appl.Physiol. 1970 29 82-86
- III VELLAR, ODD D and LARS HERMANSEN Physical performance and hematological parameters. With special reference to hemoglobin and maximal oxygen uptake.
Acta med.Scand. 1971 189 Suppl. 522. 1-40
- IV HERMANSEN LARS and MILENA WACHTLOVA, Capillary density of skeletal muscle in well-trained and untrained men
J.appl.Physiol. 1971 30. 860-863

In the text these publications will be referred to by their Roman numerals.

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PREFACE

The work on which the present monograph is based was carried out at the Institute of Work Physiology Oslo Norway; Department of Physiology Gymnastik och idrottshögskolan Stockholm Sweden; and the Institute of Hygiene University of Oslo Oslo Norway.

An investigation such as the one reported in this monograph is often the result of extensive and stimulating teamwork. It was also the case in this instance and there are several persons to whom I wish to express my sincere gratitude.

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I also wish to express my gratitude to Professor Kristian Lange Andersen who first introduced me into this field of physiology.

Finally it is the author's pleasure to acknowledge gratefully the help, willingness and cooperation of all of the 732 subjects who participated in these studies.

Oslo, February 1973

Lars Hermansen

CHAPTER I

GENERAL INTRODUCTION

The capacity to perform physical work has been a matter of primary interest for centuries. Clearly it must have been a subject of the utmost importance for our ancestors when survival depended upon their physical ability to provide food and adequate shelter for themselves and their families. The physical performance capacity of man is still a subject of considerable importance even for the people of today living in industrialized countries where the need for muscular work is claimed to be limited. The fact is, however, that in spite of the many labour-saving devices now available, a considerable amount of rather heavy physical work is still being done. It has been found, for instance, that a large number of the members of the Norwegian, as well as the Swedish Labour Unions regard their work as being physically heavy (Bolin *et al.* 1969, Karlson 1972).

The individual's physical performance capacity depends on a variety of factors including heredity, age, sex, training, state of health, motivation etc. The capacity to perform most ordinary daily activities, i.e. prolonged muscular work, depends on the individual's ability to transport oxygen from the air to the working cells. Under these conditions, the individual's maximal oxygen uptake is a useful indication of the over-all physical performance capacity.

The technique for measuring the over-all physical performance capacity in human subjects in terms of maximal oxygen uptake was developed by Hill and co-workers in 1924. In spite of the fact that physical performance capacity has been a subject of extensive studies for almost 50 years and that the technique originally described by Hill *et al.* (1924) has in principle remained unchanged, there are still a number of theoretical and methodological problems to be solved.

The existing methodological problems may possibly be one of the factors explaining the large differences observed in the values for maximal oxygen uptake reported by different groups in various countries (Robinson 1938, Astrand 1952, Astrand 1960, Hettlinger *et al.* 1961). However, more important are probably the differences in functional capacity due to hereditary and environmental factors, including daily levels of physical activity.

It is generally recognized that the capacity to transport oxygen to the working muscle cells is of primary importance for the individual's ability to perform muscular work. The oxygen transport in its turn depends on many interrelated functions, the relative importance of which needs to be assessed further. Of these, the functional capacity of the respiratory and the circulatory systems plays a major role.

A considerable reduction in the level of physical activity both on the job and

during leisure hours has been observed in many industrialized countries. This may conceivably affect the general level of physical performance capacity not only in adults but also in children. Knowledge of the general level of the physical performance capacity in the population is of considerable practical interest, not only as related to work and industry but also in connection with military defense, athletic standards and the maintenance of optimal health.

The purpose of the present investigation is to elucidate further some of the questions concerning the oxygen transport system in man. More specifically the aim of the present investigation may be summarized as follows

- 1 to study the respiratory and circulatory responses to treadmill and bicycle exercise and to compare the results from these two most frequently employed testing devices.
- 2 to evaluate the effect of variations in the relative and total hemoglobin content of the blood on the maximal oxygen uptake.
- 3 to study the relationship between maximal oxygen uptake and capillary density (i.e. number of capillaries/mm²) in subjects characterized by high as opposed to average maximal oxygen uptake
- 4 to describe the variation in maximal oxygen uptake in relation to sex, age and level of physical activity in samples of the Norwegian population.

In general, problem no. 1 is dealt with in Study I and Study II. Problems no. 2 and no. 3 are considered in Study III and Study IV respectively. Unpublished results are added in order to further elucidate questions raised in problems no. 1 and 4.

In the following the different problems are presented separately. The description of the subjects and the specific methods and procedures are presented separately in each chapter. However some of the methods and procedures are common in all studies, and are therefore described in the first part of the monograph.

CHAPTER II

MATERIAL AND GENERAL METHODS

Subjects

Altogether 732 individuals between 10 and 68 years of age participated in the studies. Of these, 286 were females and 446 males. All female subjects were Norwegian, except one Swedish woman who took part in Study I. The males consisted of 376 Norwegian, 66 Swedish and 4 American subjects. A further description of the subjects is given separately in each chapter of the present study and in the original papers (Studies I – IV) on which the present monograph is based.

Methods

The oxygen uptake was determined by the Douglas bag method. The subjects breathed through a respiratory valve and the expired air was collected in Douglas bags. The respiratory valve had a "dead space" of approximately 100 ml. The inner diameter of the valve, the stopcock and the tube of the Douglas bags was 30 mm. The connecting tube between the stopcock and the valve was smooth and not corrugated with an inner diameter of 35 mm. The total length of the tubes from the subject to the inside of the bag was approximately 50 cm. The resistance of the air collecting system was measured during different constant flows. The results of these measurements are presented in Fig. 2-1. The volume of the expired air collected in the Douglas bags was measured in a wet spirometer (i.e. in all experiments performed in the laboratory) which could be read with an accuracy of 0.2 liter. In the field studies (i.e. measurements of maximal oxygen uptake in the school children and in the office-workers) the volume of the expired air was measured in a dry gas meter. The gas meter was calibrated against the wet spirometer before and after the experimental period. The dry gas meter could be read with an accuracy of 0.2 liter. The gas analyses were performed on a Scholander apparatus (Scholander 1947) or on a modified Haldane apparatus (Åstrand and Saltin 1961a). The accuracy of the gas analysis when using the Scholander apparatus was determined by making 10 analyses of the same test gas. The mean value and standard deviation were 6.23 and 0.02 vol.% and 15.83 and 0.03 vol.%, for the carbon dioxide and oxygen concentrations, respectively. The accuracy of the oxygen measurements when using the Haldane gas analyzer is given by Åstrand and Saltin (1961a).

The heart rate was recorded by a conventional one channel electrocardiograph. Usually the heart rate was determined by counting the number of heart beats on 15 cm (i.e. 6 sec) of the recording paper. However in a few experiments where

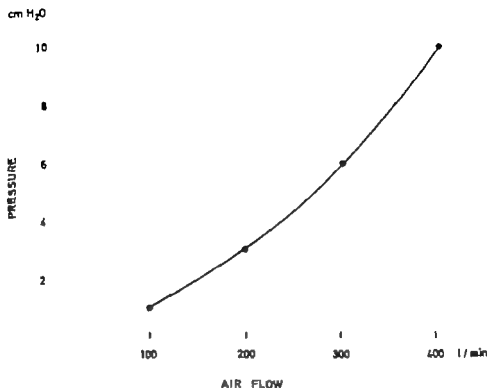


Fig. 2-1 Relationship between the valve pressure and air flow under steady state low conditions.

maximal oxygen uptake was calculated from heart rate and work load (i.e. part of Study III) the heart rate was measured by palpation over the ictus cordis or the carotid artery using a special pulse-watch i.e. the time was taken for 30 heart beats.

The blood lactate concentration was determined either by the enzymatic method as described by Scholz et al. (1959) or by the Ström modification of the colometric method of Barker and Summerson (Ström 1949). Blood samples were taken from a prewarmed clean and dry finger tip. The accuracy of the colometric method was tested by making 10 separate determinations in duplicate of the same blood sample. The mean value and standard deviation were 11.07 and 0.49 mg/100 ml, respectively (Pruett 1971).

All experiments were performed at room temperature between 19 and 21 °C, with a relative humidity of 45 to 60% and at sea level with a normal oxygen tension (i.e. 20.81 - 20.93 vol.%). The work experiments were performed on a motor-driven treadmill or on a bicycle ergometer. The bicycle exercise was performed either on a mechanically braked bicycle ergometer as described by von Döbeln (1954) or on an electrically braked Krogh bicycle ergometer (Nielsen and Frndrichsen 1938).

Procedures

The submaximal work loads, if included in the protocol normally lasted for 6–10 min. Expired air for the oxygen uptake determinations was taken during the last 1–2 min of the exercise period. Heart rate was recorded every minute both before and during the period of gas collection. The “steady state” value obtained approximately at the 6th to the 7th min, i.e. immediately before the mouthpiece and the noseclip were placed on the subject, is given in the results.

The peak blood lactate concentration was determined by taking 2 to 3 blood samples during the last minute of the exercise and/or during the first 5 minutes after the exercise had stopped.

All maximal work periods started with a 10 minute warm-up at a work load which represented approximately 50–70% of the individuals maximal oxygen uptake as calculated from the submaximal bicycle ergometer test (Åstrand and Ryhming 1954). As for the maximal work experiments on the bicycle ergometer the procedure suggested by Åstrand and Saltin (1961a) was used. Maximal oxygen

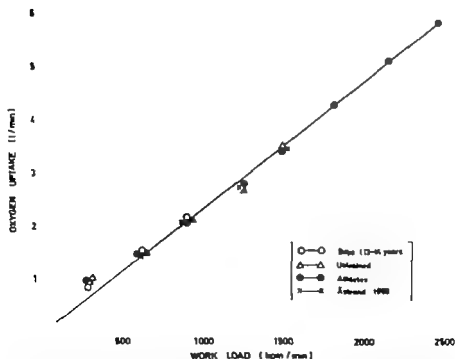


Fig. 2-2. Oxygen uptake (l/min) during submaximal bicycle exercise in untrained and well-trained adult subjects, and in boys. The work loads lasted for 10 min and the oxygen uptake measurements were performed between the 8th and the 10th min. Crosses denote results from Åstrand (1980) and open circles denote results from studies by Hermansen and Oseld (1971a).

uptake (l/min) was first predicted from the heart rate and submaximal work load on the bicycle ergometer according to the method of Åstrand and Ryhming (1954). With the aid of Fig 2-2 the work load was then chosen so as to be just high enough to reach the predicted maximal oxygen uptake. The work load for the next (second third or fourth) maximal exercise was increased by increments of 200 kpm/min. The pedal frequency during both submaximal and maximal work loads was set at 50 revolutions per min by a conventional metronome if not otherwise stated.

On the treadmill a somewhat modified procedure originally described by Taylor et al. (1955) was used. Also in this case the maximal oxygen uptake was first predicted from a submaximal test on the bicycle ergometer (Åstrand and Ryhming 1954) and expressed in ml/kg x min. With the aid of Fig 2-3 the speed for the first maximal run was chosen according to the same principles as described for the bicycle ergometer. The speed for the next maximal run was increased by increments of 2 km/hr.

The work time for the maximal exercise periods (both bicycle and treadmill) varied between 3 and 7 minutes. The work loads, both on the bicycle ergometer and the treadmill, were increased until the classical criterion with a "leveling off" was established i.e. that oxygen uptake increased less than 3% when the next maximal work load was increased by 200 kpm/min on the bicycle ergometer or 2 km/hr on the treadmill.

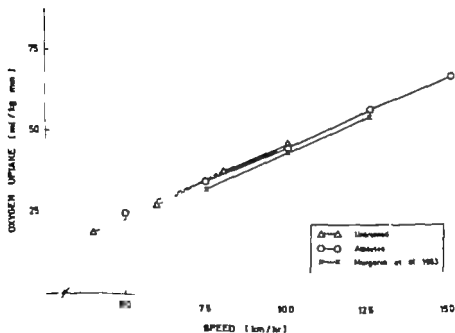


Fig. 2-3 Oxygen uptake (ml/kg x min) during submaximal treadmill exercise in untrained and well-trained subjects. Crosses denote results from Margaria et al. (1963)

The error of the maximal oxygen uptake measurements was calculated from 42 double determinations varying between 1.82 l/min and 5.57 l/min. The mean value and the standard deviation were 3.94 and 0.08 l/min, and 3.90 and 0.08 l/min for the first and second determinations, respectively. Furthermore, the reproducibility of the maximal oxygen uptake measurements was tested in one well-trained subject making 5 determinations within 13 days. The results of these experiments are given in Table 2-1.

Statistics

The arithmetic mean (\bar{x}), standard deviation (SD) and the standard error of the mean (SE) have been calculated according to standard statistical methods.

For the correlation analysis the formula for linear regression has been used. The coefficient of correlation r obtained by

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum ((x - \bar{x})^2)((y - \bar{y})^2)}}$$

and the regression equation of y and x is:

$$Y = \bar{y} + b(x - \bar{x}) = a + bX$$

where \bar{x} and \bar{y} denote the mean of the independent and the dependent variables, a is a constant and b is the regression coefficient obtained by

$$b = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sum (x - \bar{x})^2}$$

The statistical significance was tested by means of the student's t -test. The degrees of freedom were calculated from the number (n) of paired observations minus the number of 2 (i.e. $2n - 2$). The conventional level of statistical significance, $p < 0.05$ was used.

The accuracy of a single determination of the different methods was calculated in most cases from 10 separate analyses of the same sample. The error of the methods could be read from the mean value and the standard deviation of these 10 separate analyses.

Table 2-1 Individual and mean values (with standard error of the mean and standard deviation) for oxygen intake and related parameters obtained during 5 different maximal uptake determinations in the same subject within 13 days.

Examination	Maximal values			
	Oxygen uptake l/min	Heart rate beats/min	Pulmonary ventilation l/min	Blood lactate concentration mM
1st	5.57	181	244.0	10.4
2nd	5.54	181	207.3	10.3
3rd	5.49	180	215.3	10.7
4th	5.51	181	214.8	9.9
5th	5.46	181	216.0	9.8
Mean	5.51	181	219.5	10.2
SD	0.04	0.4	14.2	0.4
SE	0.02	0.2	7.1	0.2

CHAPTER III

RESPIRATORY AND CIRCULATORY RESPONSES TO DIFFERENT TYPES OF EXERCISE

Previous Investigations

In many types of muscular work there is a linear increase in oxygen uptake with increasing work load. This straight line relationship exists up to a critical level, beyond which the work load usually may be increased somewhat more, but the oxygen uptake eventually levels off or even declines (Taylor et al 1955 Mitchell et al. 1958a) This upper level of oxygen uptake is, according to Hill et al. (1924) defined as the individual's *maximal oxygen uptake*.

It was shown by Christensen (1931) and later by others (Asmussen and Hemmingsen 1958 Åstrand and Saltin 1961b Stenberg 1966) that maximal work with the legs (i.e. large muscle groups) gives higher values for oxygen uptake than maximal work with the arms (i.e. small muscle groups) Taylor et al. (1955) found a small but significant increase in the maximal oxygen uptake in one subject who was able to work an arm ergometer while running on the treadmill. Furthermore, higher values for maximal oxygen uptake have been reported during skiing than during running or bicycling (Christensen and Högberg 1950 Åstrand 1952 Andersen et al. 1961). Åstrand and Saltin (1961b) measured oxygen uptake during different types of muscular work, including maximal work performed by arms, legs, and arms plus legs on a bicycle ergometer. This investigation (Åstrand and Saltin 1961b) and later studies by Stenberg and co-workers (Stenberg 1966 Stenberg et al 1967) however did not confirm earlier observations (Christensen and Högberg 1950 Åstrand 1952 Taylor et al. 1955) showing that maximal work with arms plus legs gives higher values for oxygen uptake than those obtained during maximal exercise with legs "only" i.e. running or bicycling. Thus, according to the above cited studies it appears fairly well established that maximal exercise with the legs elicits higher values for oxygen uptake than does maximal work with the arms. However whether or not the oxygen uptake is higher during arm plus leg exercise than during leg exercise "only" is still an open question.

According to the studies cited above it appears that in order to load the respiratory and circulatory systems maximally large muscle groups must be engaged during the work. For this reason the bicycle ergometer and the treadmill are the two most frequently used laboratory testing devices for measurements of oxygen uptake and related parameters in human subjects. The testing procedures vary however from one laboratory to another and whether or not the two procedures give identical results is still an unanswered question. Åstrand (1952) for instance, found no significant difference in maximal oxygen uptake between maximal treadmill exercise (inclination 1 = 1.25%) and bicycling. Later when Åstrand and Saltin (1961b) systematically compared the oxygen uptake during

different types of maximal exercise, a 5% higher oxygen uptake was found during maximal uphill running (inclination $3^{\circ} = 5.25\%$) than during maximal bicycle exercise (50 rpm). The most pronounced difference in oxygen uptake comparing maximal work on the treadmill and on the bicycle was reported by Rowell and co-workers (Chase et al 1966, Rowell 1967). In 19 subjects they found on the average 0.60 l/min higher values on the treadmill than on the bicycle ergometer. Leveling off was used as a criterion to establish the maximal oxygen uptake on the treadmill using the procedure described by Taylor et al (1955). On the bicycle, however, they used the procedure described by Luft et al. (1963) with only one continuous exercise period. This fact may possibly explain the large difference observed in these studies. Glassford et al. (1965) using procedures very similar to those in the present investigation, reported mean values for maximal oxygen uptake which were 0.28 l/min higher during uphill running on the treadmill than during maximal bicycle exercise (50 rpm). Wyndham et al. (1966) have also demonstrated significantly higher (0.24 l/min) values for oxygen uptake during maximal treadmill exercise as compared with maximal bicycling. In view of the somewhat contradictory results in the above cited studies, it is of importance to re-examine this question. As degree and type of training as well as previous experience in maximal work tests may influence the results, different groups of subjects were studied.

Subjects

Altogether 4 female and 75 male subjects participated in the present investigation. Of these, 1 female and 37 males were included in Study I and 13 male subjects participated in Study II. The remaining 3 female and 5 male subjects took part in the experiments in which oxygen uptake and related parameters were measured during uphill running and "ski-walking" on the treadmill. A further description of the subjects is given below.

Study I included 1 female and 37 male subjects, all of whom were Swedish. The subjects were divided into 7 different groups according to age and level of physical activity (i.e. state of training).

- Group A: consisted of 8 endurance athletes (21–27 years old). Their average maximal oxygen uptake was 72.7 ml/kg x min.
- Group B: included 5 trained male students (mean age 22.0 years). Their average maximal oxygen uptake was 62.2 ml/kg x min.
- Group C: consisted of 6 untrained male students (mean age was 25.2 years) with an average maximal oxygen uptake of 49.1 ml/kg x min.
- Group D: consisted of 5 male subjects from the laboratory staff. Their mean age was 30.0 years and their average maximal oxygen uptake was 61.3 ml/kg x min.
- Group E: included 10 young athletes in cross-country running (orienteering) with an average age of 22.8 years. The mean value for maximal oxygen uptake in this group was 70.3 ml/kg x min.
- Group F: consisted of 14 older athletes, still active in orienteering, age 45–68. The mean value for the maximal oxygen uptake in this group was 54.7 ml/kg x min.

Group G: Included 7 sedentary men (age 37–50 years) who were studied after 2 months of physical training (i.e. cross-country running). Their average maximal oxygen uptake was 45.9 ml/kg x min.

Mean values for age, height and weight of the different groups are given in Study 1. One female and 2 additional male subjects were used to study the effects of variations in the pedal frequencies on the oxygen uptake during maximal bicycle exercise.

Study II included 13 healthy male students who took part in the hemodynamic studies. Of these, 4 were American college students, aged 19–21 years. The mean value for maximal oxygen uptake in this group was 53.8 ml/kg x min. Only one of the subjects in this group was well-trained. In addition 9 well-trained Swedish subjects, with a mean maximal oxygen uptake of 67.4 ml/kg x min were included. Five of these 9 subjects competed regularly in endurance events. All subjects were familiar with the testing procedure. Individual values for age, height and weight are given in Study II.

In addition 3 female and 5 male well-trained subjects participated in the experiments with "ski-walking" and running on the treadmill. The mean values for age, height and weight of the 5 male subjects were 25.4 years, 180.4 cm and 73.2 kg, respectively. The corresponding mean values for the 3 female subjects were 25.3 years, 171.3 cm and 62.3 kg. The mean values for maximal oxygen uptake were 73.9 ml/kg x min in the male subjects and 56.9 ml/kg x min in the female subjects.

Methods and Procedures

Cardiac output was determined by the dye-dilution method using the same technique and procedures as described by Saltin et al. (1968) and Ekblom (1969). The error of the method was about 3–6% (Saltin et al. 1968; Ekblom 1969).

Arterial blood pressure was measured with the strain gauge system (Statam P23db or Elema, EMT 490) just before the cardiac output measurements were performed. The oxygen content of the blood was determined by the van Slyke technique or spectrophotometrically according to the method of Holmgren and Pernow (1959).

The cardiac output determinations were performed between the 6th and the 8th minute of exercise. During the maximal exercise the measurements were done during the last 2 min of exercise. Arterial blood pressure was measured just before the cardiac output determinations. Blood samples for measurements of oxygen content and blood lactate concentration were taken during the last minute of exercise. From the simultaneous (or almost simultaneous) measurements of cardiac output, blood pressure, oxygen uptake, heart rate and oxygen content of the arterial blood, the arteriovenous oxygen difference, peripheral resistance and the oxygen content of the mixed venous blood were calculated.

The "ski-walking" was performed on a large treadmill (3.3 meter long and 1.0 meter wide) with an inclination of 12° (21%). The speed of the treadmill was, in these experiments, varied between 60 and 160 m/min. The subjects were walking with slightly bent knees and were using the ski poles as in skiing (i.e. imitating the skiing movements) see Fig. 3-1. In order to obtain sufficient friction between the



Fig 3-1 'Ski-walking' with poles on the treadmill.

tips of the poles and the belt of the treadmill, and without damaging the belt, the metal spikes of the ski poles were removed and replaced by larger rubber knobs.

The measurements of oxygen uptake and other related parameters during ski-walking were performed in the same way as in the experiments when running on the treadmill.

Results

Oxygen uptake and related variables during treadmill and bicycle exercise

"Ski-walking" and running

The results of the determinations of the respiratory and circulatory responses to submaximal and maximal ski-walking and running on the treadmill are presented in Fig. 3-2 and in Table 3-1. During submaximal exercise in 2 subjects the heart rate was found to be somewhat higher during running than during "ski-walking"

with poles when compared at the same metabolic rate (i.e. oxygen uptake). The difference was almost the same (i.e. approximately 10 beats/min) in both subjects, and at all work levels (Fig 3-2). The pulmonary ventilation however showed no consistent difference. There was a tendency towards higher values for the pulmonary ventilation during "ski-walking" than during running in the female subject (M.K.). However no difference was observed in the male subject (J.S.). During maximal exercise in 8 subjects (Table 3-1) the mean values for oxygen uptake were found to be 4.82 l/min during ski-walking and 4.71 l/min during uphill running. The difference, 0.11 l/min (2.3%) was statistically significant ($0.01 > p > 0.001$). The mean value for the pulmonary ventilation was also found to be higher during ski-walking compared with uphill running. The difference 12.1 l/min was statistically significant ($p < 0.001$).

In spite of the above significant differences in the highest obtained values for oxygen uptake and pulmonary ventilation during "ski-walking" and running there was no significant difference in the corresponding values for heart rate and blood lactate concentration (Table 3-1).

Treadmill and bicycle exercise

The results of the examination of the respiratory and circulatory responses to submaximal bicycle and treadmill exercise in well-trained and untrained subjects (i.e. Group A and C) are shown in Fig 2-2 and Fig 2-3 (Chapter II) and Fig 3-3 and Fig 3-4.

The mean values for oxygen uptake during submaximal bicycle and treadmill exercise (Fig 2-2 and Fig 2-3 in Chapter II) were approximately the same as those reported by other investigators (Åstrand 1960 Margaria et al 1963). Heart rate, pulmonary ventilation and blood lactate concentration were found to be somewhat higher during bicycle exercise when compared with treadmill exercise at the same metabolic rates (Fig 3-3). For example heart rate was found to be on the average 8, 10, 12 and 14 beats/min higher on the bicycle than on the treadmill at oxygen uptakes of 1.0, 1.5, 2.0 and 3.0 l/min, respectively in the untrained subjects. The corresponding values in the well-trained subjects were 5, 5, 4 and 6 beats/min. The difference in heart rate between submaximal bicycle and treadmill exercise was found to be statistically significant ($p < 0.05$). This was also the case for the blood lactate concentration. However the difference in the pulmonary ventilation between submaximal bicycle and treadmill exercise was not found to be statistically significant ($p > 0.2$). The lower heart rate during submaximal exercise on the treadmill than on the bicycle at the same absolute oxygen uptake (i.e. l/min) gave a higher oxygen pulse during treadmill exercise. This was also the case, even when the difference in the maximal oxygen uptake values for the two types of work was taken into consideration (Fig 3-4).

The results of the determination of oxygen uptake, pulmonary ventilation, heart rate and blood lactate concentration during maximal exercise on the bicycle and on the treadmill in 55 male subjects are shown in Fig 3-5 and Table 1 in the Appendix.

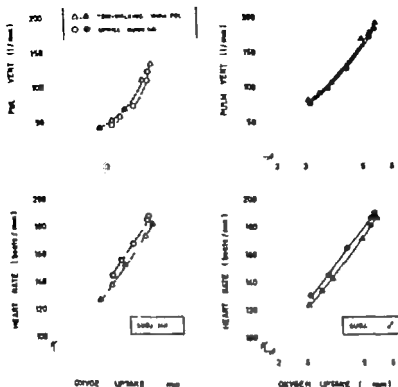


Fig 3-2. Pulmonary ventilation (upper part) and heart rate (lower part) at different work loads during "ski-walking" and running on the treadmill in one female and one male subject.

Table 3 1 Mean values, \pm standard error of the mean and standard deviation, for oxygen uptake, pulmonary ventilation, heart rate and blood lactate concentration during maximal ski-walking and maximal running (Phi 3) on the treadmill in 8 well-trained subjects.

Type of exercise	No. of subject	Maximal values			
		Oxygen uptake l/min	Pulmonary ventilation l/min	Heart rate beats/min	Blood lactate concentration mM
Uphill running	8	4.71 ± 0.36 0.96	157.5 ± 8.6 22.7	189.5 ± 2.6 7.0	11.1 ± 0.8 2.0
"ski-walking" with poles	8	4.82 ± 0.37 0.98	149.6 ± 10.2 27.0	187.6 ± 3.0 7.8	10.9 ± 0.9 2.4
Difference		$0.01 > p > 0.001$	$p < 0.001$	not signif	not signif.

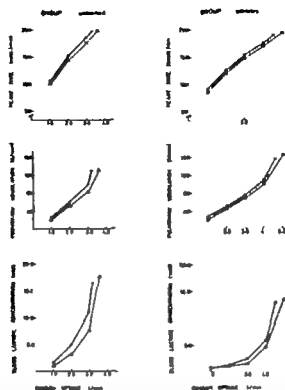


Fig. 3-3 Mean values for heart rate pulmonary ventilation and blood lactate concentration in relation to oxygen uptake during submaximal exercise on the bicycle ergometer (filled symbols) and on the treadmill (unfilled symbols) of untrained subjects (left panel) and athletes (right panel)

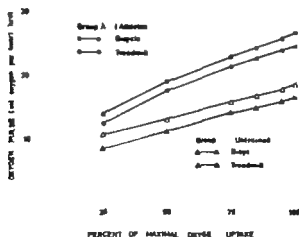


Fig 3-4. Mean values for oxygen pulse during submaximal and maximal bicycle and treadmill exercise in well trained (athletes) and untrained subjects.

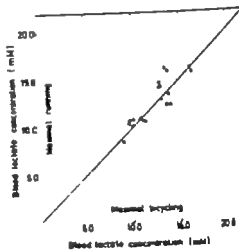
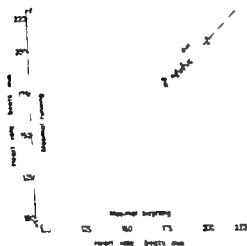
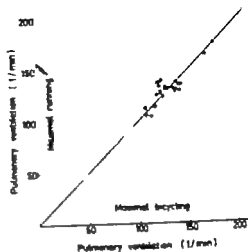
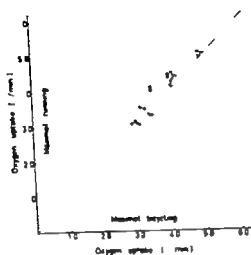


Fig 5-5 Individual values for oxygen uptake pulmonary ventilation, heart rate and blood lactate concentration during maximal bicycle exercise (50 rpm) compared with corresponding values during maximal treadmill running (inclination 5%)

As degree of training age as well as previous experience in maximal work tests might influence the results, the subjects were divided into different groups

The mean values for oxygen uptake during maximal treadmill exercise were found to be 0.41, 0.34, 0.37 and 0.31 l/min higher than the oxygen uptake during maximal bicycle exercise for groups A, B, C and D respectively (Table 1 in the Appendix)

The differences were statistically significant ($p < 0.001$). Similar results were also obtained for the groups E-G although the differences were somewhat less pronounced. The mean value for the total material (i.e. 55 subjects) for the oxygen uptake during maximal treadmill exercise was found to be 4.18 l/min. The corresponding value obtained in the same subjects during maximal bicycle exercise was 3.90 l/min. The difference, 0.28 l/min (or 7.2%) was statistically significant ($p < 0.001$). The largest individual difference between the highest oxygen uptake values obtained during maximal treadmill and bicycle exercise, 0.61 l/min (or 14.0%) was seen in one of the cross-country runners (Group A). The largest difference observed in the untrained subjects (Group C) was somewhat lower (0.49 l/min) but expressed on a percentage basis, the difference was 18.7%. On the other hand, it should also be noted that 7 of the 55 subjects had higher values during maximal bicycle exercise than during maximal treadmill exercise. The largest individual difference in this direction was 1.9%. Thus, the individual values for the difference in oxygen uptake during maximal running compared with maximal bicycle exercise varied between +18.7% to -1.9%, with a mean difference of 7.2%.

In spite of the foregoing significant difference in the highest obtained values for oxygen uptake during maximal treadmill and bicycle exercise there was no significant difference in the corresponding mean values for pulmonary ventilation, heart rate and blood lactate concentration (Fig. 3-5)

Effect of variation in the pedal frequency (bicycling) and inclination (treadmill exercise)

Since a difference in oxygen uptake between maximal bicycle and treadmill exercise with the above test procedures was observed, some supplementary experiments were undertaken in which the inclination of the treadmill and the pedal frequency on the bicycle were varied. The sequence for the bicycle and the treadmill tests was selected at random. Table 3-2 summarizes the results from the experiments in which oxygen uptake during maximal running at no inclination is compared with the oxygen uptake during maximal running, uphill (i.e. 3-4.5°). The mean value for the maximal oxygen uptake during running at no inclination was 4.48 l/min. The corresponding value during uphill running was 4.68 l/min. The difference, 0.20 l/min, was statistically significant ($p < 0.001$). The mean value for pulmonary ventilation was also found to be lower during maximal running at no inclination when compared with uphill running. The difference, 10.3 l/min (7%) was statistically significant ($p < 0.001$). In spite of the difference in oxygen uptake and pulmonary ventilation, no significant difference in the corresponding mean values for heart rate and blood lactate concentration could be observed.

The results of the experiments concerning oxygen uptake during maximal

Table 3— Individual and mean values, & standard error of the mean and standard deviation, of oxygen uptake, pulmonary ventilation, heart rate and blood lactate concentration during maximal walking at 0° inclination and uphill running (3.0–4.5°) on the treadmill.

Subjects	Maximal values							
	Oxygen uptake l/min STPD		Pulmonary ventilation l/min BTPS		Heart rate beats/min		Blood lactate concentration, mM	
	0	3.0–4.5	0	3.0–4.5	0	3.0–4.5	0	3.0–4.5
P-OA	4.28	4.37	124.8	131.7	193	186	9.8	15.1
BS	5.45	5.65	156.2	172.2	180	180	14.1	14.1
LJ1	4.28	4.47	151.7	164.9	196	194	11.2	12.6
LJ	3.88	4.09	112.4	125.2	193	190	5.4	11.6
RI1	4.51	4.69	136.5	152.6	189	189	17.1	15.1
GL	4.65	4.81	161.3	157.9	197	202	14.8	17.0
Mean	4.48	4.68	140.5	150.8	191	190	12.1	14.3
SE	0.4	0.24	8.6	8.9	7.8	3.3	1.9	0.9
SD	0.53	0.34	19.3	18.6	6.2	7.4	4.2	1.9

bicycle exercise with 50, 60 and 70 rpm are shown in Table 3-3. The mean value for oxygen uptake was found to increase from 3.96 l/min at 50 rpm to 4.06 l/min at 60 rpm. The difference of 0.1 l/min was found to be statistically significant ($p < 0.001$). However, no significant increase was demonstrated if the pedal frequency was increased to 70 rpm. The fact that there seems to be a peak value for oxygen uptake using pedal frequencies around 60–70 rpm was most markedly demonstrated in subject CS (Fig. 3-6).

Hemodynamic response to submaximal and maximal bicycle and treadmill exercise

The results of the determinations of cardiac output and related variables at rest and during exercise on the bicycle and on the treadmill are shown in Fig. 3-7 to Fig. 3-13 and Table 2 in the Appendix.

During submaximal exercise no significant difference in cardiac output was observed at a given oxygen uptake when the two work situations were compared (Fig. 3-7). However, the heart rate at a given oxygen uptake was found to be somewhat higher during submaximal bicycle exercise than during submaximal treadmill exercise. This observation is in good agreement with the results of the experiments reported in Fig. 3-4. Consequently, the stroke volume was somewhat higher during submaximal treadmill exercise than during submaximal bicycle exercise. However, when the results for heart rate and stroke volume were related to the oxygen uptake expressed in percent of the individuals' maximal oxygen uptakes obtained during bicycling and treadmill running, no significant difference was observed (Fig. 3-8). There was a considerable increase in the stroke volume

Table 3.—5. Individual and mean values, by standard error of the mean and standard deviation, for oxygen uptake, pulmonary ventilation, heart rate and blood lactate concentration during maximal bicycle exercise with 50, 60 and 70 revolutions per minute.

Subjects	Maximal values											
	Oxygen uptake			Pulmonary ventilation			Heart rate			Blood lactate		
	l/min STPD			l/min BTPS			beats/min			concentration, mM		
	50 rpm	60 rpm	70 rpm	50 rpm	60 rpm	70 rpm	50 rpm	60 rpm	70 rpm	50 rpm	60 rpm	70 rpm
ME	5.45	5.60	6.54	172.9	179.7	192.7	198	198	198	17.1	18.8	18.1
LM	4.40	4.56	4.46	165.3	186.1	181.0	191	191	191	14.8	14.6	13.1
GL	4.09	4.23	4.16	141.2	146.1	154.1	194	193	193	13.1	17.2	14.2
LH	4.06	—	4.19	168.4	—	164.9	194	—	194	15.7	—	15.3
FL	2.91	3.01	3.00	104.1	114.3	116.1	186	186	187	12.1	11.8	10.9
EA	2.86	2.91	2.82	122.5	124.1	113.6	194	194	192	9.7	11.1	12.3
Mean	3.96	4.06	4.03	144.0	144.1	148.7	193	192	193	13.8	14.7	14.0
SE	0.44	0.16	0.45	12.0	13.0	13.5	1.8	2.2	1.6	1.2	1.5	1.1
SD	0.97	1.13	1.00	26.9	26.0	30.1	4.0	4.4	3.6	2.7	3.3	2.3

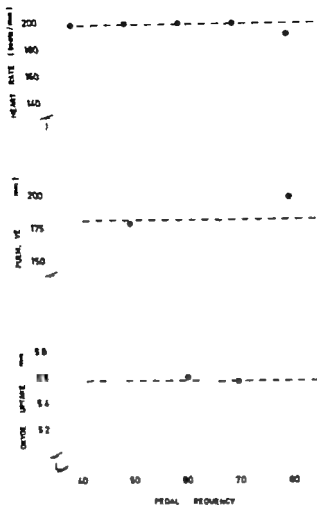


Fig. 5-6 Heart rate pulmonary ventilation and oxygen uptake during maximal bicycle exercise with different pedal frequencies (i.e. 40-80 rpm) in subjects CS. For comparison the value (dashed line) for maximal running uphill (5°) is also included. The work load was 2700 kpm/min in all experiments.

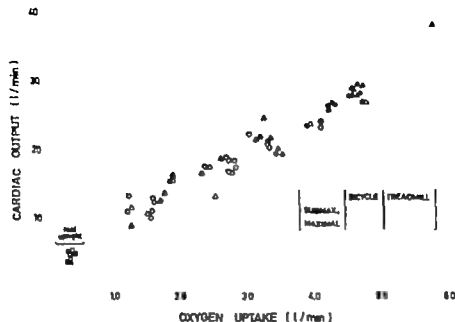


Fig. 3-7 Individual values for cardiac output in relation to oxygen uptake at rest and during submaximal and maximal bicycle and treadmill exercise.

from rest (sitting in an upright position on the bicycle ergometer) to light exercise (i.e. about 30% of the individual's maximal oxygen uptake). Beyond this work load there was a small further increase in the stroke volume with increasing work loads (Fig 3-8). Only on 4 occasions, 3 during treadmill exercise and 1 during bicycle exercise was the highest stroke volume observed during submaximal work loads. The values for the arteriovenous oxygen difference at rest and during exercise are shown in Fig 3-9. It will be seen that no consistent difference in the arteriovenous oxygen difference could be demonstrated between the two exercise forms (i.e. bicycle and treadmill). The values for the peripheral resistance, calculated from blood pressure and cardiac output, also showed no consistent difference between the bicycle and treadmill exercise (Fig 3-10).

A comparison of the hemodynamic data at maximal treadmill and bicycle exercise is shown in Fig 3-11 to Fig 3-13 and Table 2 in the Appendix.

In this study (Study II) the oxygen uptake during maximal bicycle exercise (50 rpm) was 4.31 l/min compared with 4.57 l/min during maximal treadmill exercise (inclination 3°). The mean difference 0.26 l/min was statistically significant ($p < 0.001$) and almost identical to that observed in the 55 subjects in Study I.

The mean values for cardiac output were found to be 27.5 l/min and 25.9 l/min during maximal treadmill and bicycle exercise, respectively. The difference 1.6 l/min (or 6%) was statistically significant ($p < 0.001$) and the order of magnitude was approximately the same as the difference in the mean values for maximal oxygen uptake.

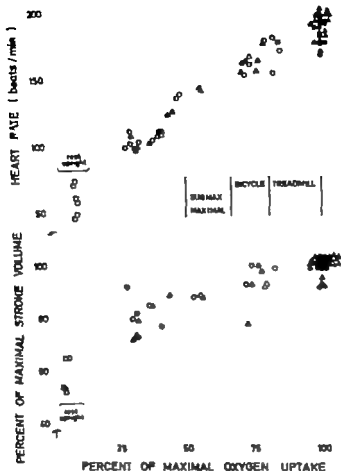


Fig. 3-8 Individual values for heart rate and stroke volume in relation to oxygen uptake expressed as percent of the individual's maximal oxygen uptake at rest and during submaximal and maximal bicycle and treadmill exercise.

The mean values for the heart rate during maximal exercise were 187 and 185 beats/min during treadmill running and bicycling, respectively. The difference was small but statistically significant ($p < 0.01$). Consequently the stroke volume was higher during maximal treadmill exercise than during maximal exercise on the bicycle ergometer. The mean difference was approximately 5% ($p < 0.01$). Thus, the stroke volume was found to be higher both during submaximal and maximal treadmill exercise than during submaximal and maximal bicycle exercise.

On the other hand, no significant difference was observed in the arteriovenous oxygen difference between the two exercise forms (Fig. 3-9 and 3-11). However both the mean arterial pressure and the peripheral resistance was found to be significantly lower during maximal treadmill than during maximal bicycle exercise.



Fig 3-9 Individual values for arteriovenous oxygen difference in relation to oxygen uptake expressed as percent of the individual's maximal oxygen uptake during submaximal and maximal bicycle and treadmill exercise.

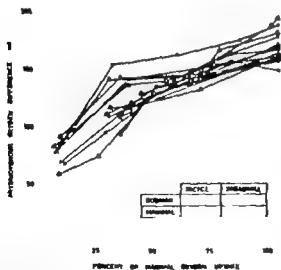


Fig 3-10. Individual values for peripheral resistance in relation to oxygen uptake during submaximal and maximal bicycle and treadmill exercise.

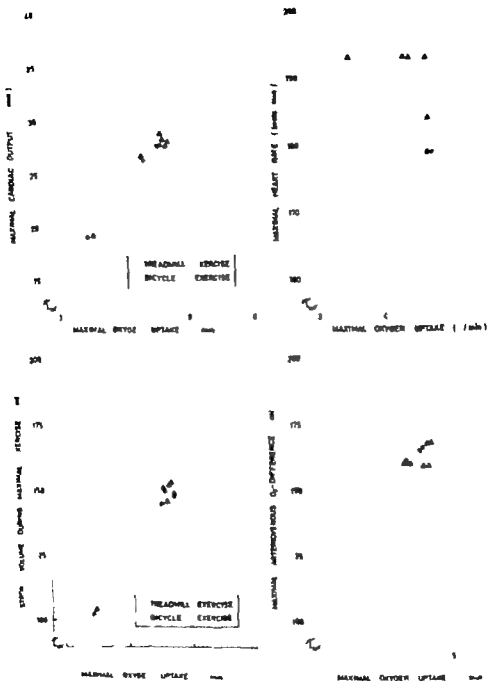


Fig 3-11 Individual values for cardiac output and heart rate (upper panel) and stroke volume and arteriovenous oxygen difference (lower panel) in relation to maximal oxygen uptake

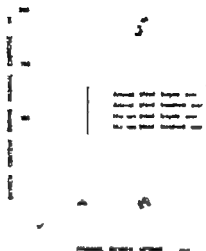


Fig. 3-12 Individual values for oxygen content in arterial blood (measured values) and in mixed venous blood (calculated values) in relation to maximal oxygen uptake.

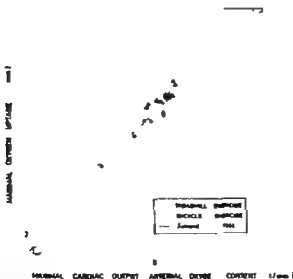


Fig. 3-13 Individual values for maximal oxygen uptake in relation to the total amount of oxygen distributed during exercise (i.e. maximal cardiac output x arterial oxygen content)

During maximal exercise with an oxygen saturation of 91–95%, the arterial oxygen content was 18.8 and 19.0 vol. % for treadmill and bicycle exercise respectively (Fig. 3–12). As the maximal cardiac output was highest on the treadmill, the amount of arterially transported oxygen during maximal exercise was also higher on the treadmill compared with bicycle exercise (Fig. 3–13). The mean values were 5.14 l/min and 4.93 l/min for the treadmill and bicycle exercise, respectively. This difference, 0.21 l/min ($p < 0.01$) is approximately the same as the difference observed for the maximal oxygen uptake in the two types of work positions.

Comments

One of the major questions to be answered in the present investigation was whether or not the same values for maximal oxygen uptake can be obtained during maximal bicycle and treadmill exercise, using two standard procedures for obtaining the maximal values in the two types of work. By applying the leveling off criterion for the estimation of maximal oxygen uptake the present investigation on 55 subjects in Study I and 13 subjects in Study II showed that oxygen uptake was 6–7% higher during maximal uphill running than during maximal bicycle exercise. It should however be noted that the individual variation was large, and the difference in oxygen uptake during maximal treadmill and bicycle exercise varied between plus 18.7% to minus 3.9%. It should also be emphasized that this variation was not related to the state of training of the subject, previous experience in maximal exercise tests, or to previous training in bicycling.

The hemodynamic studies during maximal bicycle and treadmill exercise showed that the higher oxygen uptake during treadmill exercise was explained by a higher cardiac output during running compared to bicycling. This higher cardiac output was partly explained by a somewhat higher heart rate and partly by a higher stroke volume. However in spite of the observed differences in oxygen uptake, heart rate and stroke volume, there were no significant differences in the values for pulmonary ventilation, blood lactate concentration, arteriovenous oxygen difference or peripheral resistance between maximal treadmill exercise compared with maximal bicycle exercise.

In summary it may be concluded that although there are some differences, the physiological responses to maximal treadmill and bicycle exercise are very similar. The observed differences are relatively small and thus both treadmill and bicycle may be recommended. On the other hand, the subjective feeling of fatigue or exhaustion during the maximal work necessary to obtain the maximal oxygen uptake is usually less when using the treadmill as compared to the bicycle ergometer. Furthermore, on the basis of the results from the present investigation it may also be concluded that maximal oxygen uptake is only *maximal* for a specific type of exercise. Consequently strict comparability of maximal oxygen uptake values requires strictly comparable and standardized conditions during the measurements. However if maximal oxygen uptake is measured during strictly standardized conditions, it is a highly reproducible parameter.

CHAPTER IV

RELATIVE AND TOTAL HEMOGLOBIN CONTENT OF THE BLOOD AND MAXIMAL OXYGEN UPTAKE

Previous Investigations

The relationship between the hemoglobin content of the blood and maximal oxygen uptake (or physical work capacity) has been extensively studied by several investigators (Hjellberg et al. 1949 a,b Åstrand 1952, Sjöstrand 1953 Balke et al 1954 Robbe 1958 Sproule et al. 1960 Beutler et al 1960 Cotes et al 1969 Andersen and Barkve 1970 Ericsson 1970 and others) However the studies have yielded conflicting results.

Thus, Robbe (1958) found no influence of anemia (individual values for hemoglobin concentration as low as 8.2 g/100 ml) on the physical work capacity of pregnant women. Cotes et al. (1969) made the same observation in nonpregnant anemic housewives (mean hemoglobin concentration 8.6 g/100 ml) Sproule et al (1960) on the other hand observed very low values for maximal oxygen uptake in 9 anemic patients in comparison with normal values for healthy subjects of comparable age. Several investigators (Hjellberg et al 1949a b Åstrand 1952 Sjöstrand 1953 Holmgren and Åstrand 1966) have emphasized that there is a close relationship between total hemoglobin content and maximal oxygen uptake (or physical work capacity PWC_{150}) Thus, Åstrand (1952) found that the correlation coefficient between maximal oxygen uptake and total hemoglobin content was 0.94 for the female and 0.97 for the male subjects, indicating that the total hemoglobin content might be an important determinant for maximal oxygen uptake.

A reduction in the total amount of hemoglobin caused by bleeding has been shown to cause a lowering of the physical work capacity (Karpovich and Mülman 1942 Balke et al. 1954) Rowell et al (1964) on the other hand showed that a reduction in hemoglobin concentration of 14% due to repeated phlebotomies during an eight-day period gave almost no reduction in maximal oxygen uptake.

In spite of the fact that several investigators have examined the relationship between maximal oxygen uptake and hemoglobin concentration apparently no one has studied the effect of altered hemoglobin content brought about by increasing the dietary iron intake, in a longitudinal type of study

The aim of the present investigation was to study the effect of altered hemoglobin content on the maximal oxygen uptake both in cross-sectional and longitudinal studies.

Subjects

Altogether 284 subjects participated in the study. Of these 183 were school children, 97 were physical education students and 4 were patients with iron deficiency anemia. All the 284 subjects participated in Study III which also included a supplementary group of 8 female physical education students. However as the results obtained in these subjects did not give any new information they were not included in this monograph.

School children

The school children material which included 95 boys and 88 girls was obtained from the pupils of two elementary schools (i.e. Hjeltefjord and Bjornevatn) in Finnmark. Pertinent data describing the subjects are given in Study III.

Physical education students

47 female and 50 male students participated in the study. The mean age was 24.5 years for the men and 22.5 years for the women. The mean values for maximal oxygen uptake (calculated from heart rate and work load during submaximal bicycle exercise) and hemoglobin concentration for the male students at the start of the experiment were 47.5 ml/kg \times min and 15.1 g/100 ml, respectively. The corresponding values for the female students were 40.8 ml/kg \times min and 12.91 g/100 ml. Further information about the subjects is given in Study III.

Selected cases of iron deficiency anemia

During the period of March – December 1969 four cases of iron deficiency anemia were found suitable for examination of the maximal oxygen uptake (i.e. maximal running on the treadmill). Pertinent data describing these patients are given in Table 4-1.

Methods and Procedures

The determination of the hemoglobin concentration in the present study was performed by the cyanmethemoglobin method (Larsen 1966). The accuracy of the method was tested by taking 10 separate determinations of the same blood sample. The mean value and standard deviation of the 10 analyses were 13.3 and 0.3 g/100 ml.

The total amount of hemoglobin was calculated from the determinations of hemoglobin concentration and the blood volume. The blood volume was calculated from measurements of the hematocrit and the plasma volume. The hematocrit was measured in heparinized capillary tubes after centrifugation in an hematocrit centrifuge. The accuracy of the hematocrit measurements was tested in the same way as for the hemoglobin concentration. The mean value and the standard deviation were 43.1 and 0.9%. The plasma volume was measured by the radiiodine plasma protein method of Williams and Fine (1961). The error of the method was not determined.

The experiments involving school children were performed between October 1968 and April 1969. The first examination in October 1968 included 72 school

Table 4-1 Pertinent data describing the anemic subjects.

Subjects	sex	age	height	weight	Hb concentration (g/100 ml) at start	Follow up period (weeks)	Treatment
T.O.H. (blood donor)	male	45	182	86	14.2	13	iron
T.H. (petr. pract. patient)	female	33	171	58.2	10.9	7	iron
R.L. (blood donor)	female	35	170	57.9	11.0	20	iron
S.H. (blood donor)	female	18	165	59.5	11.4	26	placebo 4 weeks iron 22 weeks

children (i.e. 39 boys and 33 girls). The second (final) examination was performed on the same subjects in April 1969. However, in this final examination an additional material of 56 boys and 53 girls was included. Thus, the final examination included altogether 183 subjects, i.e. 95 boys and 88 girls. The hemoglobin concentration was measured on the same day and prior to the maximal oxygen uptake determinations. Maximal oxygen uptake was determined by the Douglas bag method in all school children both at the first and the second examination. No iron medication was given to the school children.

The experiments on the physical education students were performed between August 1967 and May 1968. According to the results from the measurements of hemoglobin concentration at the start of the experiment and to the criteria of normal hemoglobin values in Norwegian men and women (Natvig et al. 1967) the subjects were divided into two groups:

Group A: with normal hemoglobin values

Group B: with subnormal hemoglobin values

By paired sampling technique all subjects in Group A (i.e. with normal Hb values) were divided into two subgroups:

Group 1: the iron therapy group

Group 2: the placebo group

All subjects in Group A and Group B were given iron or placebo tablets. Iron was given in the form of ferrofumarate. One iron tablet was equivalent to approximately 60 mg of bivalent iron. The iron tablets were of identical shape and size and labelled "ferrofumarate X" and ferrofumarate Y" respectively. Each person received the tablets packed in boxes of 100 and was instructed to take one tablet once a day regardless of the blood values at the start of the experiment. The tablets were taken in the evening. Re-examination of the hemoglobin concentration and

maximal oxygen uptake was performed with approximately eight weeks intervals, the last one in May 1968. Including the first examination six examinations were made altogether. The actual consumption of iron was recorded at the end of the experimental period (i.e. after 40 weeks). Maximal oxygen uptake was calculated from heart rate and work load during submaximal bicycle exercise according to the method of Astrand and Ryhming (1954) in all female and male students. However maximal oxygen uptake was also measured using the Douglas bag method, in a sample of 20 male and 21 female students at the first examination (i.e. Aug. 1968) and in 16 male and 15 female students at the sixth examination (i.e. May 1969). Total hemoglobin content was measured in 20 students at the first examination (Aug. 1968) and in 22 students at the sixth examination (May 1969).

The anemic patients were studied for periods of 7 to 26 weeks. Maximal oxygen uptake and hemoglobin concentration were measured at several re-examinations in the same patient. The interval between the re-examinations was usually about one week but varied between 3 and 44 days. All determinations of maximal oxygen uptake in the anemic subjects were performed using the Douglas bag method. None of the subjects donated blood during the period of investigation. Iron tablets were administered to 3 of the patients from the start of the experiments. The dose was 2-3 tablets a day. The 4th subject received placebo tablets during the first 4 weeks. From then on however the placebo tablets were replaced by iron tablets. Otherwise, the scheme of administration was similar to that described for the students.

Results

Cross-sectional analysis

The results of the determination of hemoglobin concentration and maximal oxygen uptake in the various age groups of the school children are given in Fig. 4-1. The mean values for the hemoglobin concentration were fairly well in accordance with the proposed normal values of Natvig et al. (1967) for school children of comparable age. In some groups, however the mean values were below the previously proposed standards. The values for maximal oxygen uptake were, on the average slightly higher than the values given by Astrand (1952) for school children in Stockholm of comparable age.

It will be seen from Fig. 4-1 that the mean values for maximal oxygen uptake in the boys increased almost linearly with increasing age from the age of 10 to 14 years. From then on, the maximal oxygen uptake continued to increase although at a somewhat slower rate. The mean values for the hemoglobin concentration on the other hand showed no systematic changes in the age groups 10-13 years. However from the age of 13 to 16 years, the hemoglobin concentration (mean values) increased from 13.2 to 14.5 g/100 ml. In the girls, the maximal oxygen uptake showed a marked increase from 10 to 13 years. From then on, no change in maximal oxygen uptake in relation to increasing age was observed. The mean values for the hemoglobin concentration in the schoolgirls showed no systematic changes

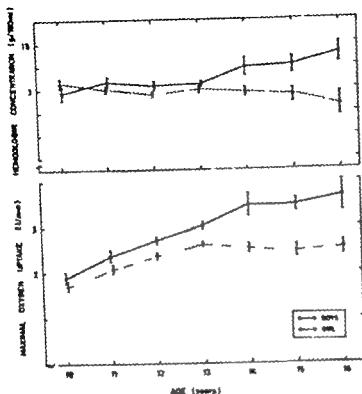


Fig. 4-1 Mean values (\pm SE) for hemoglobin concentration and maximal oxygen uptake in various age groups of school children.

with increasing age. However the hemoglobin concentration (mean values) were somewhat higher for the 10 year-old girls and somewhat lower for the 16 year-old girls than for the other age groups examined.

The correlation between hemoglobin concentration and maximal oxygen uptake was tested in two sets of values. The results from the first examinations on school children and students were pooled and designated the *1st examination* although the investigations in the two groups were undertaken at different calendar times. In the same way the results obtained in the final examinations in both school children and students were pooled and designated the *2nd examination*. The results obtained in each of the examinations have been analyzed separately. Thus, the correlation coefficients (see Table 3 in the Appendix) refer to one set of values in each individual. It should also be emphasized that the correlation coefficients given in Table 3 in the Appendix are based on the *measured* (i.e. Douglas bag method) values for maximal oxygen uptake only both in the student and the school children material.

There was a statistically significant positive correlation between the hemoglobin concentration and the maximal oxygen uptake (l/min) both at the 1st and the 2nd

examination. However when the data for maximal oxygen uptake were expressed in ml/kg x min no definite statistical relationship could be demonstrated. At the first examination where 41 students and 68 school children were studied, the correlation coefficient was 0.07 i.e. not significant. However in the second examination a statistically significant relationship was found ($p < 0.001$). It should be noted however that the number of school children investigated had been markedly increased from the first to the second examination (i.e. from 68 to 177).

In order to make further analysis of the relationship between hemoglobin concentration and maximal oxygen uptake the correlation coefficients in the various subgroups (male and female students, schoolboys and schoolgirls) have also been calculated. With two exceptions, none of the subgroup correlation coefficients were statistically significant. The general finding is further illustrated in Fig 4-2 where corresponding values for hemoglobin concentration and maximal oxygen uptake (l/min) for the students and school children at the first examination are given (i.e. total material). It is evident that, although the scatter of the individual values around the calculated regression line is considerable, the correlation between the two parameters is positive and significant ($r = +0.67$). However within the four subgroups, which are encircled in the figure, there was no definite correlation between the two parameters. Although there is a wide scatter and a great deal of

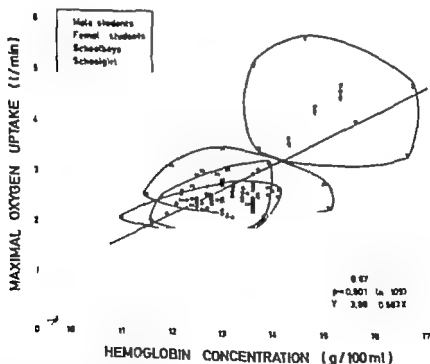


Fig 4-2 Corresponding values for hemoglobin concentration and maximal oxygen uptake. The values in each of the subgroups (i.e. schoolgirls, schoolboys, female students and male students) are encircled.

overlapping in the individual values in the various subgroups, there is a tendency to a concomitant increase in both hemoglobin concentration and maximal oxygen uptake from subgroup to subgroup in the following order schoolgirls, schoolboys, female students and male students. A similar covariation between the changes in hemoglobin concentration and maximal oxygen uptake may also explain the finding of a positive correlation in the school children (i.e. schoolgirls, first examination and schoolboys, second examination) In both cases, small individuals are compared with others who were nearly full-grown.

The relationship between total amount of hemoglobin and maximal oxygen uptake in 20 students at the first examination (i.e. Aug. 1968) and 22 students at the second examination (i.e. May 1969) was found to be statistically significant in both examinations, ($p < 0.001$) However the close correlation which was observed in the total student material (i.e. both female and male students) could not be confirmed when each sex was examined separately. The same lack of relationship was found when the correlation coefficients were based on the maximal oxygen uptake values in ml/kg x min (i.e. after correction for differences in body weight). However the number of subjects in each group was small.

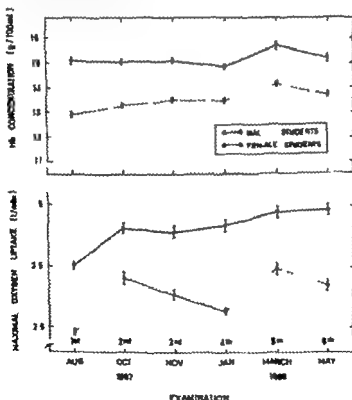


Fig. 4-5 Mean values (\pm SE) for hemoglobin concentration and maximal oxygen uptake in female and male students during the course of the experimental period

Longitudinal analysis

The results of hemoglobin concentration and maximal oxygen uptake from the six examinations in the students are represented in Fig. 4-3.

Although there were some fluctuations in the values from examination to examination there was a pronounced increase in maximal oxygen uptake during the 9-month examination period both in the male and female students. The mean increase in maximal oxygen uptake from the first to the final examination was approximately 12 ml/kg x min in both the male and female students, representing an increase of 25 and 31% respectively. It should however be noted that these values for maximal oxygen uptake were not measured directly but calculated from heart rate (and work load) during submaximal exercise on the bicycle.

The hemoglobin concentration also showed a small increase (i.e. 7% over the initial value) during the same period in the female students. However the mean values for hemoglobin concentration in the male students remained almost unchanged during the whole examination period (i.e. 9 months).

The results of the studies concerning the effect of iron supplements on the hemoglobin concentration and maximal oxygen uptake are presented in Fig. 4-4. It will be noted that the subgroups within each sex were similar both with regard to the hemoglobin concentration (with exception of the subnormal group) and maximal oxygen uptake at the start of the experimental period.

The same increase in maximal oxygen uptake as shown in all male and all female subjects (Fig. 4-3) was also found in the different subgroups. Although there were some fluctuations in the values, no systematic difference between the different subgroups could be demonstrated. The hemoglobin concentration increased in all female subjects, regardless of whether iron or placebo tablets were consumed. The largest increase was observed in the subnormal group. A similar increase however was not found in the male subgroups. Furthermore from the results in Fig. 4-4 it is evident that the maximal oxygen uptake may increase and the hemoglobin concentration decrease or vice versa, from one examination to another.

The assessment of the actual consumption of tablets (iron and placebo) at the end of the experiment revealed that the subjects had followed the instructions to take one tablet a day during the 40-week experimental period. The average consumption per individual was 270 tablets, and thus in good agreement with the proposed consumption according to the plan of the investigation i.e. approximately 280 tablets.

Selected cases of iron deficiency anemia

The results of the studies concerning the relationship between hemoglobin concentration and maximal oxygen uptake in 4 selected cases of iron deficiency anemia are given in Fig. 4-5. All subjects were given iron tablets during the whole experimental period with the exception of subject III H who received placebo tablets for 4 weeks and from then on iron treatment was given according to the same scheme as for the 3 other subjects. In two subjects (i.e. To.Hc. and T.H.) there was a pronounced increase in the hemoglobin concentration. Maximal oxygen uptake also showed a slight increase, but only from the first to the second

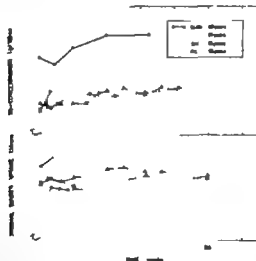
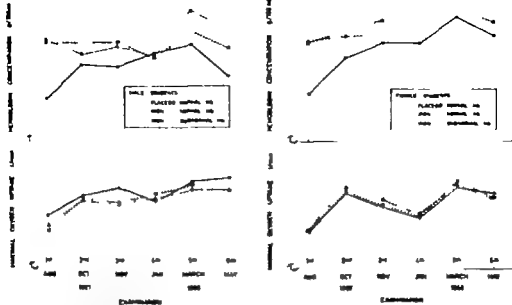


Fig. 4-5 Individual values for hemoglobin concentration and maximal oxygen uptake in 4 anemic patients during the course of the experimental period. All subjects were given iron tablets (i.e. 100-180 mg per day) during the whole experimental period. The only exception was S.H. who received placebo tablets for 4 weeks. From then on iron medication was given according to the same scheme as for the 3 other subjects.

examination. From then on, the values for maximal oxygen uptake remained almost unchanged. For the two other subjects (i.e. S H and R L) both hemoglobin concentration and maximal oxygen uptake varied during the course of the 20-24-week examination period. However, no definite relationship seemed to exist between the variations of the two parameters examined.

Comments

From the results of the present investigation it is evident that the values for both relative and total hemoglobin content vary considerably from one individual to another and also from time to time in the same individual. It should also be emphasized that the variations in the hemoglobin concentration were approximately the same in the placebo and the iron therapy group. A crucial question is of course whether or not the students followed the instructions for taking the iron tablets given by the investigators. However, the assessment of the actual consumption of tablets (iron and placebo at the end of the experiment) revealed that the subjects had followed the instructions fairly well. The average consumption per individual during the experimental period was about 270 tablets, and this was in good agreement with the theoretical consumption according to the plan of the experiment (i.e. approximately 280 tablets).

A corresponding large variation was also observed in the values for maximal oxygen uptake. The most pronounced variation was observed in the female subjects who showed a marked increase from the first to the second examination (Fig. 4-4). During this period the students were subjected to regular outdoor training (cross-country running and field exercises). However, during the period from October to January the activity program was changed to indoor activities (i.e. gymnastics). During this time a pronounced fall in maximal oxygen uptake was observed. The 5th examination was performed in March, immediately after a period with outdoor activities (i.e. skiing). The variations in the male subjects were smaller, although they followed approximately the same pattern as for the female subjects.

In the student material most of the values for maximal oxygen uptake were estimated by the indirect Åstrand Ryhming method (Åstrand and Ryhming 1954). Although this method does not provide the same degree of accuracy as the direct Douglas bag method, it is reasonable to believe that it may give valuable information about the longitudinal changes as each individual serves as his own control. Furthermore, the pronounced increase in maximal oxygen uptake found in the female students from the first to the second examination was confirmed in a group of 8 students studied with Douglas bag method. The mean increase in maximal oxygen uptake of the 47 female physical education students was 0.90 l/min from the first to the second examination. The corresponding increase in the 8 female students studied with the Douglas bag method was 0.57 l/min. Thus, it seems unlikely that methodological errors may account for more than a fraction of the observed total variations in the maximal oxygen uptake during the 9-month experimental period. It should also be emphasized that with regard to the correlation analysis, only values for maximal oxygen uptake determined with the Douglas bag method were used.

CAPILLARY DENSITY OF SKELETAL MUSCLE
AND MAXIMAL OXYGEN UPTAKE

Previous Investigations

The main function of the capillaries is to distribute oxygen, nutritive materials and hormones to the various cells of the body and to collect end products of the metabolism for further transportation to the excretory organs.

The capillary density is essential for all of these functions. For instance analyses of the oxygen transport in peripheral tissues require information about the differences in the pO_2 of the various compartments between the erythrocytes and the mitochondria. Direct measurements of the pO_2 gradients have been difficult to obtain, and several indirect methods have been developed. The classical approach to the problem has been to calculate the pO_2 gradients from the diffusion coefficient for oxygen in the tissue, the metabolic rate and the dimensions of the capillary bed (Krogh 1929). However these calculations are only as good as the data on which they are based, the number of capillaries per mm^2 being one of the most important parameters. From the studies of Petréu et al 1937 Vannotti and Pfister 1933 Vannotti and Magiday 1934 it has been assumed that the increase in physical performance after training was associated with an increase in the number of capillaries per mm^2 in the trained muscle. However results for measurements of the capillary density in human skeletal muscle in connection with bed rest and training (Saltin et al. 1968) do not support this view. Thus, morphological studies in animals and human subjects concerning the capillary density in well-trained and untrained muscle have yielded conflicting results.

Subjects

Altogether 23 male subjects, 13 athletes and 10 untrained students participated in the study. Their age varied between 22–32 years. Of these, 7 athletes and 8 students were included in Study IV. In addition 6 athletes and 2 students were studied. However due to technical difficulties the results of these subjects (i.e. 5 athletes and 2 students) were not as complete as the material presented in Study IV. In the primary material (7 athletes and 8 students) biopsy samples from the lateral portion of the quadriceps femoris muscle were obtained both before and after maximal exercise. In the additional material samples were obtained either before or after the work. All athletes had been engaged in hard physical training and competition for at least 5 years. All of them participated in endurance events, i.e. marathon running, orienteering and bicycling (road racing). They were all successful athletes of high national standard. Their average maximal oxygen uptake

was 71.3 ml/kg x min ($n = 13$). The student material included a total of 10 subjects. None of the students had been engaged in any regular physical training during the last 3 years. Their average maximal oxygen uptake was 52 ± 8 ml/kg x min ($n = 10$).

Methods and Procedures

The capillary density was determined in small biopsy specimens (10–20 mg wet weight) from the lateral portion of the quadriceps muscle. The biopsies were performed according to the method of Bergström (1962). The tissue specimens were immediately (i.e. within 5–10 sec after the biopsy was finished) immersed in 10% formalin. After a fixation period of one month or more the specimens were treated according to standard histological procedures (i.e. immersed in alcohol and embedded in paraffin). After sectioning the periodic-acid Schiff (PAS) reaction according to the method of Hecht (1958) was used. The number of capillaries and muscle fibres were counted in a microscope with a vertical camera. Twenty fields were calculated from each specimen using 18–20 different sections. The size of the field used for all calculations was $40\,000\ \mu^2$. The capillaries and muscle fibres were only calculated in cross-section. From these two measurements two other parameters were calculated, i.e. the capillary/fibre ratio and the diffusion distance according to the method described by Krogh (1919). The diffusion distance is defined as the average half distance between two capillaries on the cross-section.

Two biopsies were performed in each subject. The first biopsy was taken after the subject had been resting in the laboratory for 20–30 min. The last one was taken immediately (i.e. within 5–10 sec) after the cessation of 4–6 min maximal exercise on the treadmill. The maximal oxygen uptake was determined on separate days.

Results

Individual values for the number of capillaries per mm^2 and the number of muscle fibres per mm^2 in small muscle specimens obtained in the lateral portion of the quadriceps femoris muscle and maximal oxygen uptake in athletes and students are given in Fig. 5-1 and Fig. 5-2. It appears that (Fig. 5-1) although the scatter of the individual values is quite large the number of capillaries per mm^2 in the untrained students and in the well-trained athletes were approximately the same. The mean values were 579 and 587 capillaries per mm^2 in the students ($n = 10$) and the athletes ($n = 13$) respectively. Statistical analysis revealed no significant difference. However as will be seen from Fig. 5-2 the number of muscle fibres per mm^2 was smaller in well-trained than in untrained subjects. Thus, the average size of the muscle fibres in the well-trained subjects was approximately 30% larger than in the untrained muscle. The difference was statistically significant ($p < 0.001$).

Since the number of muscle fibres per mm^2 was lower in the well-trained than in the untrained group and the number of capillaries per mm^2 was the same in both groups, the capillary/fibre ratio was found to be significantly higher ($p < 0.05$) in the well-trained group. On the average each muscle fibre from the well-trained

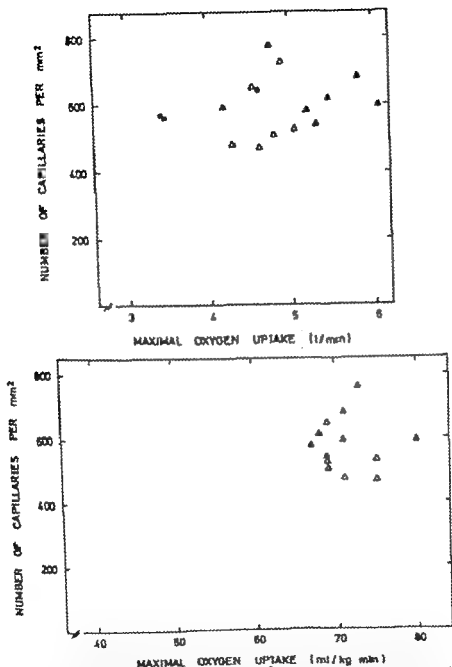


Fig. 5-1 The number of capillaries per mm² in small muscle specimens obtained from the lateral portion of the quadriceps femoris muscle of athletes (Δ, Δ) and untrained students (\circ, \circ) in relation to the individuals maximal oxygen uptake expressed as l/min (upper panel) and ml/kg x min (lower panel). The filled symbols denote mean values from samples obtained before and after maximal exercise. The unfilled circles and triangles denote single values obtained from samples taken before or after maximal exercise.

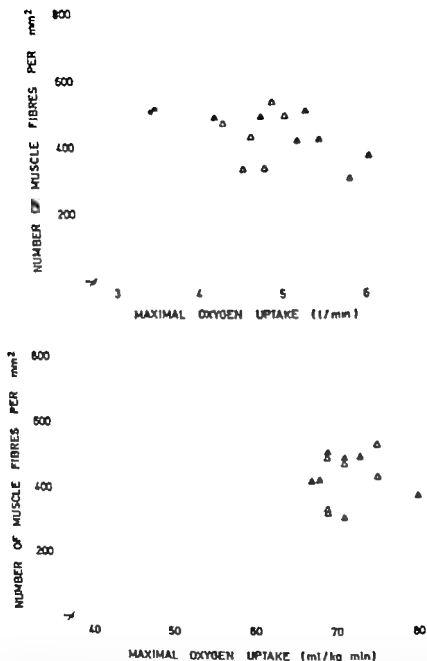


Fig. 5-2. The number of muscle fibres per mm² in small muscle specimens obtained from the lateral portion of the quadriceps femoris muscle of athletes (Δ) and untrained students (○) in relation to the individuals maximal oxygen uptake expressed as l/min (upper panel) and in ml/kg x min (lower panel). For explanation of the symbols see Fig. 5-1.

muscle was supplied with 1.5 capillaries, whereas in the untrained muscle the ratio capillaries to fibres was approximately 1.

However, when the diffusion distance, i.e. average half distance between the capillaries, was calculated according to the method of Krogh (1929) no statistically significant difference between the well-trained and untrained muscle was observed.

Comments

The capillary density of heart and skeletal muscle has been studied by several investigators, mainly by means of infusion techniques or by counting of erythrocyte-packed capillaries (for references see Landis and Pappenheimer 1963, Hammersen 1968). These methods have, however, been subject to criticism (Hort 1968) since not all capillaries are necessarily filled with ink or packed with red blood cells. However, the method used in the present investigation, i.e. the PAS staining reaction, is considered to be specific to the polysaccharides in the basement membrane of the capillaries. Thus, with this method it should be possible to count all capillaries, whether packed with erythrocytes or not, or whether the capillaries are open or closed.

In order to make a further evaluation of the method for estimating capillary density in the present investigation, muscle samples were obtained at rest and immediately after maximal exercise in the same subjects. According to Krogh (1929) the number of open capillaries in the skeletal muscle is markedly increased during exercise. Although there is a rapid fall in the muscle blood flow after cessation of work (Grimby et al. 1967) the blood flow during the first 5–10 sec of the recovery period is still very high compared with resting values. Normally all biopsies were completed during this time period. Thus, when the first biopsy was performed only a few capillaries were open, whereas when the second biopsy was taken the number of open capillaries was much higher. The results obtained both in the well-trained and untrained subjects, showed that there was no statistically significant difference between the values for capillary density in samples taken before or immediately after the maximal exercise. Consequently, the method used in the present investigation may be considered to give a fairly reliable estimation of the number of capillaries per mm^2 in the muscle samples of the two groups of subjects in the present investigation.

The number of capillaries per mm^2 in the present study was found to vary between approximately 400–800 (average about 600 capillaries per mm^2) both in the well-trained and in the untrained subjects. Capillary counts based on an infusion technique or counting of erythrocyte-packed capillaries led to estimates in the range of 200–600 capillaries per mm^2 in resting skeletal muscle and to 600–5000 capillaries per mm^2 for working skeletal muscle (Landis and Pappenheimer 1963). On the other hand, values of 150–200 capillaries per mm^2 are reported in other studies using electron microscopic methods or by counting of capillaries in frozen sections of skeletal muscle (Landis and Pappenheimer 1963, Saltin et al. 1968).

This large variation in the number of capillaries per mm² may at least partly be explained by the fact that almost all ordinary histological techniques produce some shrinkage of the muscle tissue. Consequently direct comparison of the absolute values for capillary density from one study to another may be of limited value. However in the present study the same fixation and staining procedures were used for all tissue specimens, both from the well trained and untrained muscles. Therefore the shrinkage is considered to be approximately the same in both groups of subjects.

CHAPTER VI

MAXIMAL OXYGEN UPTAKE IN NORWEGIAN SUBJECTS

Previous Investigations

Maximal oxygen uptake differs greatly from one individual to another (Robinson 1938). The value is dependent upon the body weight or more accurately the muscle mass of the subjects (Boskirk and Taylor 1957). Maximal oxygen uptake is also dependent upon the individual's level of physical activity (Åstrand 1952, Hermansen and Andersen 1963, Saltin and Åstrand 1967). Furthermore, maximal oxygen uptake first increases and later decreases with increasing age (Åstrand 1952, Åstrand 1960, Andersen et al. 1961) and is lower in female than in male subjects (Åstrand 1952, Åstrand 1960). Most studies describing the variations in maximal oxygen uptake with sex and age have been performed as cross-sectional studies. Apparently no one has examined these variations in a longitudinal type of study. Consequently it is not known whether or not the results obtained in cross-sectional and longitudinal studies are comparable.

The variation in maximal oxygen uptake with sex, age and level of physical activity are rather well established in some countries, such as Sweden (Åstrand 1952, Åstrand 1960, Saltin and Åstrand 1967) and U.S.A. (Robinson 1938, Mitchell et al. 1958a, Hettinger et al. 1961, Rodahl et al. 1961, Knuttgen 1967). Although some reports on maximal oxygen uptake in samples of the Norwegian population are already published (Andersen et al. 1961, Hermansen and Andersen 1963, Andersen and Hermansen 1965) it is of interest to give a more complete picture of the variations with sex, age and level of physical activity. Both cross-sectional and longitudinal studies are included.

Subjects

Altogether 593 subjects are included in this study. The material consists of school children, untrained and trained students, office-workers and different groups of well-trained athletes.

The results of the school children are part of a larger study (to be published) dealing with the problems of the relationship between maximal oxygen uptake and the body dimensions. However, although this question may also be regarded as important when describing the changes in maximal oxygen uptake with sex, age and level of physical activity, it is not included in the present publication.

School children

The school children material consisted of pupils from 3 different elementary schools in the county of Finnmark i.e. the schools in Kjøllefjord Båtsfjord and Bjørnevåtn. Altogether 308 school children 142 girls and 166 boys were included in the material. The height, weight and number of subjects in the various age groups are given in Table 4 in the Appendix. Twenty-eight female and 30 male pupils from the Bjørnevåtn school were studied twice a year (i.e. April and October) during the period from April 1968 until April 1970. Thus altogether 6 examinations were performed in each of these subjects. The 58 subjects were sampled from two school classes i.e. 13 female and 16 male subjects from class A, and 15 female and 14 male subjects from another class (class B). Further information regarding these subjects is given in Table 5 and Table 6 in the Appendix.

Students

The student material included a total of 75 individuals. Of these 21 females and 20 males were physical education students. These subjects were also included in Study III. The remainder of the student material 34 subjects, were all untrained and none of these took part in any regular physical activity. Pertinent data describing the subjects are given in Table 7 in the Appendix.

Office-workers

The material consisted of 30 females and 57 males. This group of subjects was studied in 1961 being part of the material included in a cand real thesis at Oslo University (Hermansen 1964). None of these subjects were well-trained, although some of them regularly took part in outdoor activities, i.e. cross-country skiing, swimming etc. Data characterizing the subjects are given in Table 7 in the Appendix.

Athletes

Altogether 123 athletes were included in the material. Of these, 88 were male and 35 were female athletes. All subjects were athletes of high national standard and most of them belonged to the Norwegian National Team in the different sports events. Some of them had been participants in several World Championships and Olympic Games. These studies were performed during the period from 1967 to 1971. Pertinent data describing the subjects are presented in Table 8 and Table 9 in the Appendix.

Results

Cross-sectional and longitudinal studies

The results of the cross-sectional and longitudinal studies concerning the changes in maximal oxygen uptake of schoolboys and schoolgirls are given in Fig 6-1 and Fig 6-2. The longitudinal studies, which included subjects from two school classes (class A and class B) showed that the variations in the mean values for maximal oxygen with age were almost the same in the two groups of subjects (i.e. school classes). Statistical analysis revealed no significant differences.

It will be seen from Fig 6-1 and Fig 6-2 that the mean values for maximal oxygen uptake in the longitudinal studies were approximately the same as those obtained in the cross-sectional studies. The differences were small and not statistically significant. It should be emphasized that the number of subjects investigated in the cross-sectional studies varied from one age group to another. Furthermore, the number of subjects in the longitudinal studies was greater in some age groups and lower in others than was the case in the cross-sectional studies.

Variation with sex and age

In order to study the variation in maximal oxygen uptake and related parameters (i.e. pulmonary ventilation, heart rate) with sex and age in Norwegian subjects, a total of 429 untrained healthy subjects (i.e. 186 females and 243 males) were examined. The results are presented in Fig. 6-3 to Fig 6-10 and in Table 4 and Table 7 in the Appendix.

As shown in Fig. 6-3 there is a pronounced increase in maximal oxygen uptake (l/min) during childhood in both sexes. In the female subjects, maximal oxygen uptake reached a peak value at the age of 14 to 16 years. From then on, an almost rectilinear decrease with increasing age was observed. The maximal oxygen uptake was found to decrease from 2.62 l/min at the age of 16 to 1.81 l/min at the age of 54 years, i.e. 0.81 l/min (or 31%) in 38 years. Hence, the maximal oxygen uptake in the female subjects decreased approximately 0.02 l/min (or 0.8%) per year. In the male subjects, a somewhat different relationship between maximal oxygen uptake and age was observed. The maximal oxygen uptake was found to increase almost rectilinearly from the age of 11 to the age of 16 years. From then on, the maximal oxygen uptake increased at a slower rate, reaching a peak value at an age of approximately 25 years. Thereafter, gradual decrease, as for the females, was observed. Maximal oxygen uptake decreased from 3.96 l/min at the age of 25 years to 2.58 at the age of 54 years, i.e. 1.38 l/min (or 35%) in 29 years, representing about 0.06 l/min (or 1.5%) per year. Thus, the annual decrease in maximal oxygen uptake with increasing age in the male subjects was almost twice as large as that of the female subjects.

The mean values for maximal oxygen uptake in the male subjects were higher than those of the female subjects in all age groups. The mean difference in the three youngest age groups (i.e. 11, 12 and 13 years) was approximately 7%. However, due to a very large interindividual variation in both sexes, the difference was not statistically significant. The difference in the mean values for maximal oxygen

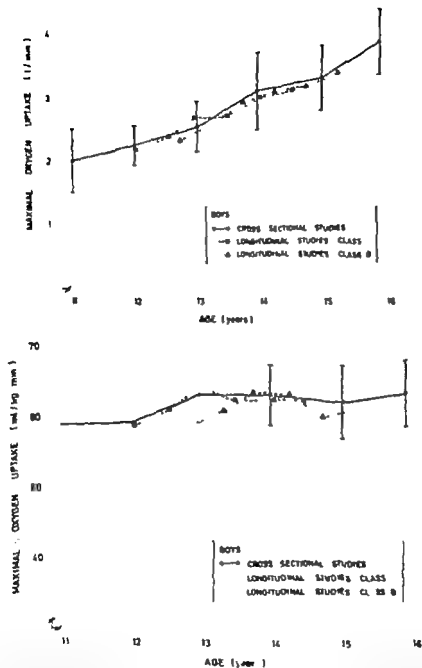
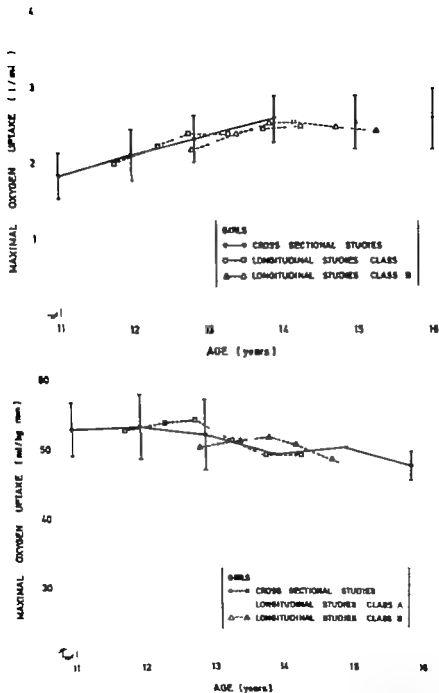


Fig. 6-1 Mean values for maximal oxygen uptake l/min (upper panel) and ml/kg \times min (lower panel) in different age groups of schoolboys obtained in cross-sectional and longitudinal studies. Vertical bars denote \pm one standard deviation.



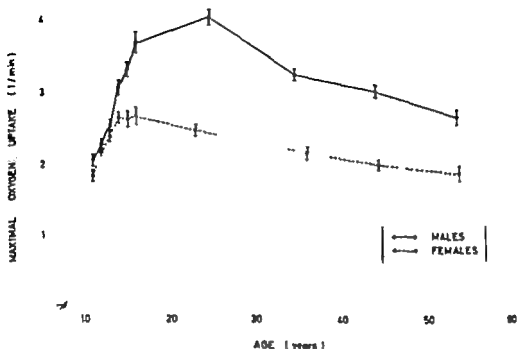


Fig. 6-5 Mean values for maximal oxygen uptake (l/min) in different age groups of female and male subjects. Vertical bars denote \pm standard error of the mean.

Uptake in the three next age groups, i.e. 14-15 and 16 years was 15.5%, 21.4% and 31.9%, respectively. The difference between the two sexes in the adult age groups was even larger. The mean values for maximal oxygen uptake were 39.1%, 33.7%, 33.4% and 29.8% lower in the female than in the male subjects in the age groups 20-29, 30-39, 40-49, 50-59 years, respectively. All these differences were statistically significant.

It should be emphasized that the maximal oxygen uptake and the body weight varied considerably from one individual to another. The large variation in these two parameters was most markedly demonstrated in the age group with the largest number of subjects (Fig. 6-4) i.e. 12 year-old boys and girls. Maximal oxygen uptake was in these subjects found to vary between 1.48 to 3.13 l/min. The body weight varied between 27.6 and 61.4 kg.

The variation in maximal oxygen uptake after correction for differences in body weight showed a somewhat different picture than that described above for the maximal oxygen uptake in l/min. The maximal oxygen uptake (ml/kg \times min) in the female subjects reached a peak value at the age of 12 years. From then on, a gradual decrease with increasing age was observed. For the male subjects, however, the peak value was observed at the age of 13 years and the values were practically unchanged in the age groups 14-15 and 16 years. However, the mean values for the male

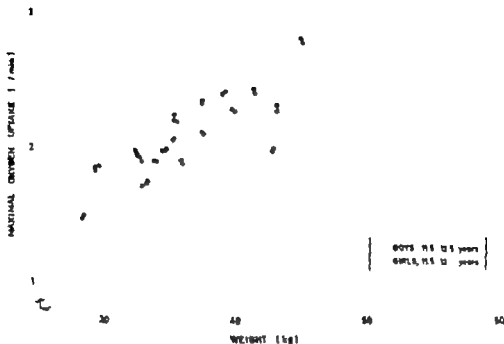


Fig. 6-4. Individual values for maximal oxygen uptake (l/min) of schoolboys and schoolgirls, 11.5 to 12.5 years of age in relation to body weight.

students were significantly lower than for the 16 year-old boys. In the case of the women, the mean values for maximal oxygen uptake continued to decrease with increasing age.

It should also be noted that the rate of decrease in maximal oxygen uptake was different in young (i.e. 12 to 23 years in the women, and 16 to 34 years in the men) and older subjects. For the female subjects the maximal oxygen uptake decreased from approximately 54 ml/kg x min at the age of 12 to about 38 ml/kg x min at the age of 23 i.e. 16 ml/kg x min (or about 30%) in 11 years. Hence the rate of decrease was approximately 2.5% per year. For the male subjects, maximal oxygen uptake was found to decrease from approximately 63 ml/kg x min at the age of 16 to about 41 ml/kg x min at the age of 34 years, i.e. 22 ml/kg x min (or 35%) in 18 years. Thus the rate of decrease in the young male subjects was approximately the same as that observed in the young women (i.e. 2% per year).

The rate of decrease in maximal oxygen uptake in the older subjects was, however, lower than in the younger subjects. The mean values for maximal oxygen uptake in the female subjects decreased from approximately 38 ml/kg x min at the age of 23 to about 29 ml/kg x min at the age of 34 years, i.e. 9 ml/kg x min, or 24% in 11 years (0.8% per year). In the male subjects, the mean values decreased from

approximately $42 \text{ ml/kg} \times \text{min}$ at the age of 34 years to $36 \text{ ml/kg} \times \text{min}$ at the age of 54 years, i.e. $8 \text{ ml/kg} \times \text{min}$, or 19% in 20 years (0.9% per year). Hence the rate of the decrease in maximal oxygen uptake with increasing age in the older subjects was found to be almost the same in both female and male subjects. Furthermore, from the results presented in Fig. 6-5 it seems that the rate of decrease in the mean values for maximal oxygen uptake has two phases: a rapid phase in the younger subjects, and a slower one in the older subjects.

In spite of the large variation in maximal oxygen uptake between female and male subjects, no significant difference in the values for heart rate during maximal exercise was observed. However the mean values were found to decrease with increasing age in both sexes (Fig. 6-6). The average decrease from the age of approximately 15 to 55 years (i.e. 40 years) was 33 and 27 beats/min, i.e. 0.83 and 0.68 beats/min each year for the females and the males respectively. The changes in the mean values for pulmonary ventilation during maximal exercise and the ventilatory equivalent (i.e. ventilation per liter of oxygen consumed) are given in Fig. 6-7 and Fig. 6-8 respectively. The variations in the maximal pulmonary ventilation were closely related to the changes in the maximal oxygen uptake and consequently the ventilatory quotient (Fig. 6-8) changed very little with age. A small decrease, however in the ventilatory quotient with increasing age was observed.

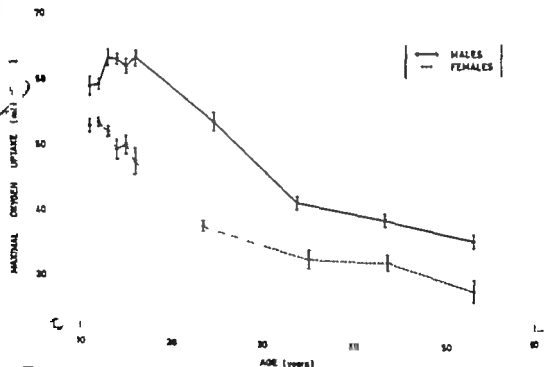


Fig. 6-5 Mean values for maximal oxygen uptake ($\text{ml/kg} \times \text{min}$) in different age groups of male and female subjects. Vertical bars denote \pm standard error of the mean.

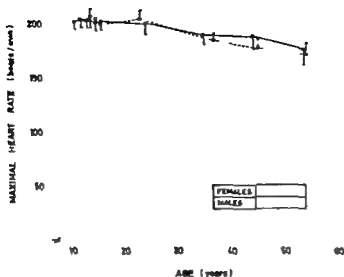


Fig. 6-6. Mean values for heart rate (beats/min) during maximal exercise in different age groups of male and female subjects.

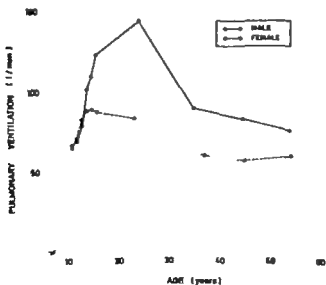


Fig. 6-7. Mean values for pulmonary ventilation (l/min BTPS) during maximal exercise (i.e. running or bicycling) in different age groups of male and female subjects.

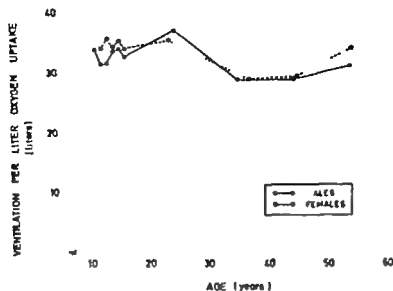


Fig. 6-8 Mean values for the pulmonary ventilation per liter of oxygen consumed during maximal exercise (i.e. running or bicycling) in different age groups of male and female subjects.

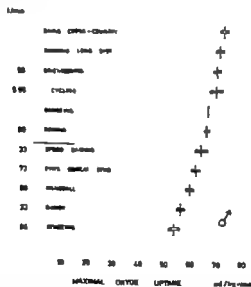


Fig. 6-9 Mean values for maximal oxygen uptake in ml/kg x min of male athletes in different sports events. The horizontal small bars at the top of the large bars denote standard error of the mean. To the left mean values for maximal oxygen uptake in l/min are also given. Mean values for maximal oxygen uptake of untrained students are also included.

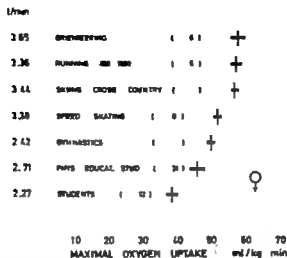


Fig. 6-10. Mean values for maximal oxygen uptake in ml/kg x min of female athletes in different sports events. The horizontal small bars at the end of the large bars denote standard error of the mean. To the left mean values for maximal oxygen uptake in l/min are also given. Mean values for maximal oxygen uptake of untrained students are also included.

Maximal oxygen uptake in athletes

In order to obtain an idea of the upper limit of the values for maximal oxygen uptake in Norwegian subjects, groups of female and male athletes in different sports events were examined. The results are presented in Fig. 6-9 and Fig. 6-10 and in Table 8 and Table 9 in the Appendix.

It will be seen that there was a large difference in the mean values for the different groups, even in well-trained subjects. The highest values were observed in athletes who took part in endurance events (i.e. cross-country skiing, orienteering, long distance running or bicycling). There were 6 male subjects with a maximal oxygen uptake of more than 6 l/min. The highest value for maximal oxygen uptake in the present investigation 6.64 l/min (72 ml/kg x min) was obtained in a top speed skater (B.T.) who came third in 1500 m speed skating at the Olympic Games in Grenoble 1968. The second highest value 6.26 l/min (67 ml/kg x min) was observed in a rower (F.H.) who was one of the two sculler rowers who came second in the Olympic Games in Munich 1972. Subject F.S.H., a cross-country runner (orienteering) of high national standard had a maximal oxygen uptake of 82 ml/kg x min. He was the only subject in the present investigation who exceeded 80 ml/kg x min.

The highest maximal oxygen uptake in the female athletes was found in two cross-country skiers, both belonging to the team which won the gold medal in the relay race (3 x 5 km) at the Olympic Games in Grenoble 1968. B.M.L. had a

The value for maximal oxygen uptake is dependent upon the body size of the subject. Consequently the height and weight of the subjects are important parameters when deciding whether or not the material is representative. However recent studies of height and weight of representative samples of the Norwegian population are not available. The control material of school children was sampled from 9 different schools in Oslo (i.e. Oslo and suburbs). The investigation was performed by Hermansen and Skaset in 1970 (unpublished results) and included a total of 1076 students between 7 and 18 years of age. The number of subjects in most age groups (i.e. one year) was approximately 50 male and 50 female subjects. The untrained adult subjects were studied in connection with the X-ray examination in the county of Ostfold (i.e. the southeastern part of Norway) in 1965 (Waalder 1972). This material included approximately 2500 male and female subjects in each age group (i.e. 5 years). Unfortunately the values for standard deviation and standard error of the mean were not calculated in this study.

The mean values for height and weight in the different groups of subjects in the present investigation are compared with the values obtained in the above mentioned studies (Hermansen and Skaset 1970, Waalder 1972) (Fig. 6-11 and Fig. 6-12). The height/weight relationship in the same subjects is shown in Fig. 6-13. It will be seen (Fig. 6-11 and Fig. 6-12) that the mean values for height and weight are somewhat lower in the school children from Finnmark than in school children of comparable age from Oslo. However statistical analyses revealed no significant differences. There was also a tendency toward somewhat higher values for the body weight at a certain height in the school children from Finnmark compared with school children in Oslo. The differences, however, were small and not statistically significant. Furthermore it will also be seen (Fig. 6-11 and Fig. 6-12) that in the adult subjects the mean values for body weight were somewhat lower and the mean values for the body height were somewhat higher in the subjects of the present investigation compared with the results obtained by Waalder (1972). The height of the athletes was approximately the same as for the untrained subjects, while the mean values for the body weight were somewhat higher in most groups of athletes, compared with untrained subjects. The mean value for the body weight of the female gymnasts, however, was lower than in untrained subjects of comparable age.

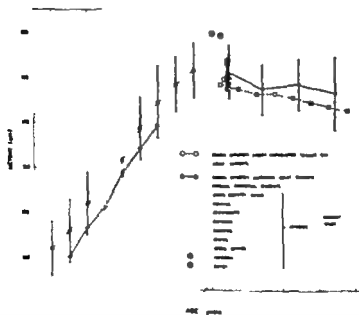
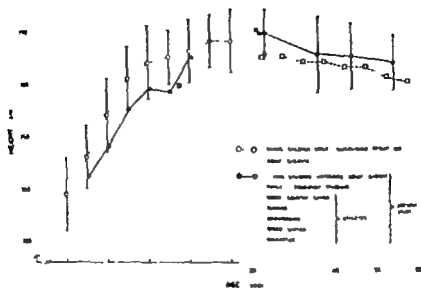


Fig. 6-11 Mean values for the body height in relation to age in female subjects (upper panel) and male subjects (lower panel) in the present investigation. Corresponding mean values for school children in Oslo (o—o) and adult subjects (□—□) are given for comparison. Vertical lines denote \pm one standard deviation.

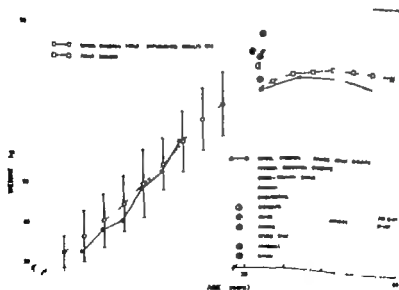
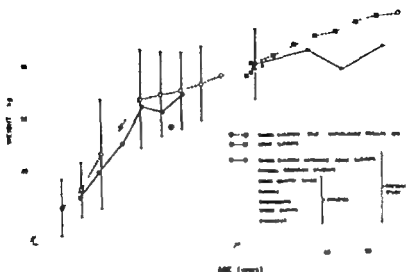


Fig. 6-12. Mean values for the body weight in relation to age in female subjects (upper panel) and male subjects (lower panel) in the present investigation. Corresponding mean values for school children in Oslo (o-o) and adult subjects (□-□) are given for comparison. Vertical lines denote \pm one standard deviation.

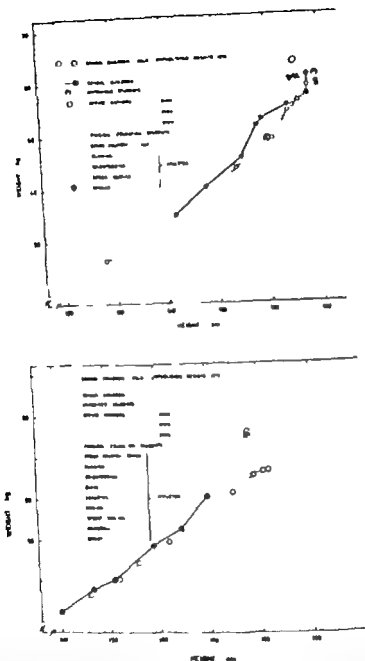


Fig 6-15 The height/weight relationship in female subjects (upper panel) and male subjects (lower panel) in the present investigation. Corresponding values for school children in Oslo (o-o) are given for comparison.

CHAPTER VII

GENERAL DISCUSSION

Maximal oxygen uptake expressed in liters per minute is a measure of the maximal rate of aerobic energy liberation within the body (i.e. the maximal aerobic power). It is also a measure of the functional capacity of the respiratory and circulatory systems (Taylor et al. 1955 Mitchell et al. 1958a Rowell 1962 Saltin et al. 1968). Maximal oxygen uptake expressed in milliliters per kilogram body weight is a measure of the maximal amount of oxidative energy available to move one kilogram of the body weight from one place to another in a certain length of time. Maximal oxygen uptake is also widely used as a measure of physical fitness. A great number of factors are known to cause a decrease in maximal oxygen uptake (Åstrand 1967). The only factors which have been shown to increase the maximal oxygen uptake are physical training (Robinson and Harmon 1941 Knehr et al. 1942 Saltin et al. 1968, Ekblom et al. 1968) and an increase of the oxygen tension in the inspired air (Margaritis et al. 1961 Wyndham et al. 1970). In a recent study by Ekblom et al. (1972) maximal oxygen uptake was also reported to increase after increasing the red cell volume by reinfusion of red blood cells.

Investigations of various groups of subjects in many countries have shown that the mean values for maximal oxygen uptake may vary considerably from one individual to another and also from one group of subjects to another (Robinson 1938 Åstrand 1952 Mitchell et al. 1958a, Åstrand 1960 Hettinger et al. 1961 Rodahl et al. 1961 Andersen and Hermansen 1965 Hermansen and Andersen 1965 Binkhorst et al. 1966 Sprynarova 1966 Andersen 1967b Cumming 1967 Ekblom and Gjessing 1968 Ikai et al. 1970 Davies 1972 Davies et al. 1972, Dehn and Bruce 1972 Eriksson 1972 Ikai and Kitagawa 1972 and others). In tables 7-1 to 7-4 the mean values for maximal oxygen uptake ($\text{ml/kg} \times \text{min}$) in the present investigation are compared with results obtained in some other countries. It will be seen from Tables 7-1 and 7-2 that the mean values for maximal oxygen uptake in the school children of the present investigation are higher than in all other studies reported in the literature, although the study by Åstrand (1952) of school children in Stockholm reported almost the same mean values in most age groups of both sexes as those obtained in the present investigation. With regard to the maximal oxygen uptake in boys (Table 7-1) it is worth noticing that the mean values obtained in USA (Robinson 1938) Canada (Cumming 1967) and Japan (Ikai et al. 1970) are almost identical. However all these values are significantly lower than the values for the Scandinavian school children (Åstrand 1952, present investigation). The lowest values for maximal oxygen uptake in boys are found in the USA (Rodahl et al. 1961). The mean

Table 7-1. Mean values for maximal oxygen uptake (ml/kg x min) in different age groups of boys from various populations.

Age years	U.S.A. Robinson 1938	SWEDEN Astrand 1952	U.S.A. Rodahl et al. 1961	CANADA Cumming 1967	JAPAN Ikai et al. 1970	NORWAY present study
11	8	57	—	—	52	59
1	50	56	32	47	53	59
13	48	57	—	—	5	63
14	47	59	33	49	51	63
15	48	60	—	—	50	62
16	50	59	32	52	55	63

Table 7-2. Mean values for maximal oxygen uptake (ml/kg x min) in different age groups of girls from various populations.

Age years	SWEDEN Astrand 1952	U.S.A. Rodahl et al. 1961	CANADA Cumming 1967	JAPAN Ikai et al. 1970	NORWAY Present study
11	53	—	—	46	53
12	51	30	42	44	54
13	49	—	—	43	52
14	48	34	38	38	50
15	45	—	—	40	50
16	47	23	39	36	48

Table 7-3. Mean values for maximal oxygen uptake (ml/kg min) in different age groups of male subjects from various populations.

Age years	U.S.A. Robinson 1938	U.S.A. Mitchell et al. 1958a	SWEDEN Astrand 1960	U.S.A. Hettinger et al. 1961	CANADA Cumming 1967	NORWAY Present study
20-29	65	45	62	30	44	54
30-39	41	39	40	29	38	42
40-49	40	35	39	26	38	39
50-59	37	32	33	23	36	36

Table 7-4. Mean values for maximal oxygen uptake (ml/kg x min) in different age groups of Swedish and Norwegian women

Age years	SWEDEN	NORWAY
	Åstrand 1960	Present study
20-29	40	38
30-39	37	34
40-49	33	35
50-59	28	29

for maximal oxygen uptake of the girls in the present investigation were somewhat higher than those obtained in girls of comparable age in Stockholm (Åstrand 1952). However as in the case of the boys, the mean values for the maximal oxygen uptake in the Scandinavian girls were found to be significantly higher than the values obtained in Canada (Cumming 1967) and Japan (Ikai et al 1970). On the other hand the mean values from the two latter studies in Canada and Japan are significantly higher than the corresponding mean values obtained in American girls (Rodahl et al. 1961).

In table 7-3 and Table 7-4 the mean values for maximal oxygen uptake of adult subjects in the present investigation are compared with corresponding mean values from some other investigations. Considering the possible differences in methodology number of subjects investigated in the various age groups of the different studies, and the time difference (the earliest study was performed in 1938) surprisingly small differences exist between samples of the male population in Boston, Stockholm, Dallas, Winnipeg and Oslo. The Philadelphia group (USA) studied by Hettinger et al (1961) is the only group in which significantly lower values than those of the above mentioned studies are reported. Only scanty information is available concerning maximal oxygen uptake in adult female subjects. The mean values for Swedish females (Åstrand 1960) are almost identical to the mean values obtained in the present study.

Although the mean values for maximal oxygen uptake are very similar in many groups of subjects from different countries, it is evident from the results presented above (Table 7-1 to 7-4) that large differences exist between the highest and the lowest values reported in the literature both for children and adult subjects. The most pronounced difference is found between the values reported by Rodahl et al. (1961) and those of the present study for 16 year-old subjects (boys and girls). The average maximal oxygen uptake in the American 16 year-old boys was 32 ml/kg x min, compared with 63 ml/kg x min in the present investigation. The corresponding value for 16 year-old American girls was 23 ml/kg x min and for the Norwegian girls the value was 48 ml/kg x min.

It is not possible, on the basis of the results of the present investigation to explain the large differences between the mean values for maximal oxygen uptake in the various groups investigated. It is also difficult to explain the large intragroup variability.

With regard to the differences in the mean values for maximal oxygen uptake as shown in Table 7-1 to Table 7-4 it should be emphasized that only a small number of subjects were studied in the various age groups of the different investigations. Furthermore most of the studies were not based on a representative sample of the population investigated. Thus there might be some doubts as to whether or not the mean values are representative for the population from which the subjects were selected. On the other hand it was shown by Grimby et al. (1970) that the mean value for maximal oxygen uptake in a large and representative group of 54 year-old male subjects was almost the same as the mean values obtained in many other studies with only a few subjects investigated. These authors (Grimby et al. 1970) concluded that the results of their study with representative sampling increased the validity of the conclusions which might be drawn from earlier studies with no representative sampling.

In the studies of Andersen et al. (1961) Hermansen and Ekblom (1966) and Ekblom and Gjesing (1968) it was shown that the mean values for maximal oxygen uptake in the nomadic Lapps in North Norway were higher in most age groups, both in the female and male subjects, than the corresponding mean values for the inhabitants of the Easter Islands (the Pascuans). On the basis of these results it might be questioned as to whether or not part of the observed differences in maximal oxygen uptake between groups of subjects from different countries (Tables 7-1 to 7-4) could be due to environmental factors or to racial differences brought about by the process of natural selection. However studies by Robinson et al. (1941) on white and American negro sharecroppers revealed no significant difference. Rodahl et al. (1961) studying white and nonwhite school children in Philadelphia found no evidence suggesting a systematic racial difference in physical work capacity. Recent studies by Davies et al. (1972) also seem to indicate that if differences between ethnic groups do exist they are certainly smaller than the large interindividual variations observed in most studies.

It is, however well known that variations in the level of physical activity may influence the maximal oxygen uptake to a large extent (Robinson and Harmon 1941 Knehr et al. 1942 Saltin et al. 1968). Thus, it is reasonable to suggest that part of the differences in maximal oxygen uptake as shown in Tables 7-1 to 7-4 must be attributed to variations in the level of physical activity. Variations in the methods and procedures employed for the determination of the maximal oxygen uptake are another factor which may probably explain part of the observed differences. With regard to the large differences in the individual values for maximal oxygen uptake within the groups, variations in the level of physical activity are probably one important factor (Saltin et al. 1968). However according to the investigations on monozygous and dizygous twins by Kiliouras (1971) the intragroup variability is almost entirely genetically determined.

Several factors are known to influence the obtained values for maximal oxygen uptake in human subjects (Rowell 1962 Åstrand 1967). The type of work used for the determination is one essential factor (Taylor et al. 1955) and the mass of muscles activated during the work is another important factor (Christensen 1931 Asmussen and Hemmingsen 1958 Åstrand and Saltin 1961b Stenberg et al. 1967). Since the treadmill and the bicycle are the two most frequently employed ergometers, it is important to compare the physiological responses to exercise using

these two testing devices. In the present investigation it was shown that "ski-walking" with poles on the treadmill yielded higher values for maximal oxygen uptake than those obtained during uphill running. Uphill running (inclination 3° or more) on the other hand gave higher values for maximal oxygen uptake than those obtained during maximal bicycle exercise (50 rpm). It was also shown that the values for oxygen uptake during maximal uphill running were higher than those obtained during maximal running at no inclination. The differences in maximal oxygen uptake between uphill running, on the one hand and bicycling and running at no inclination on the other were almost the same.

The present investigation also showed that the pedal frequency during the bicycling, and the step frequency during treadmill running are factors which modify the results to a certain extent. Maximal oxygen uptake was found to be higher during bicycling at 60 and 70 rpm than during bicycling with pedal frequencies of 40 or 50 rpm. A pedal frequency of 80 rpm however seemed to be too high to obtain the highest possible oxygen uptake. The step frequency during treadmill running at no inclination is about 190-200 steps/min. If the inclination of the treadmill is increased to about 3° the step frequency is reduced to approximately 160 steps/min. Thus, both during bicycling and running the contraction frequency of the working muscles seems to be a factor which influences the maximal oxygen uptake.

The finding of the present investigation indicating a higher oxygen uptake during arm plus leg exercise confirm earlier observations by Åstrand (1952) and on one subject studied earlier by Christensen and Høgberg (1950) showing that skiing with poles gave higher values for oxygen uptake than those obtained during running. Taylor et al. (1955) found a significant increase in maximal oxygen uptake in one subject, who while running on the treadmill also worked an arm ergometer. Furthermore Andersen et al. (1961) found higher values for maximal oxygen uptake during skiing than during bicycling. In a recent study by Kamon and Pandolf (1972) laddermill climbing (i.e. arm and leg exercise) was found to give higher values for maximal oxygen uptake than those obtained during bicycling. The results of the present investigation are also in agreement with the studies by Faulkner et al. (1971) and Miyamura and Honda (1972). All these investigations seem to indicate that the values for maximal oxygen uptake increase with the increase of the mass of muscles activated during work.

The explanation of the above described differences is a clear one. However it has been known for a long time that the motor units in human skeletal muscle vary widely in size. Moreover the full significance of these observations did not become apparent until after the investigation by Henneman et al. (1965) indicating that the excitability of the units is an inverse function of their size. Furthermore, it has been shown that human skeletal muscle consists of two different fibre types. These fibres have been classified as type I and type II (Dubowitz and Pearce 1960; Morris 1968) red and white fibres (Edström and Nyström 1969; Edström and Ekblom 1972) or slow twitch (ST) and fast twitch (FT) fibres (Gollnick et al. 1972). It is also known that the different fibre types vary with regard to both contractile and metabolic properties (Barnard et al. 1971; Burke et al. 1971). Due to the differences in the excitability of the motor units, and to differences in contractile and metabolic properties, it has been assumed (Jorfeldt 1970; Karlsson 1971) that it is mainly the

small motoneurons (α 2) and slow twitch fibres (i.e. red muscle fibres) which are activated during exercise at low work intensities and that the contribution of large motoneurons (α 1) and fast twitch fibres (i.e. white muscle fibres) increases with increasing work loads. It is conceivable that the total number of motor units, and probably also the total number of red muscle fibres activated are larger during for instance "ski-walking" than during bicycling.

If it is assumed that the muscle mass ratio between the arms and shoulders, and the legs and hips is approximately 1:6 a 15 percent increase in the maximal oxygen uptake during arm plus leg exercise should be expected. The increase observed in the present investigation was, however, only 2.3 percent. Although the muscles of the arms and shoulders are also activated to a certain extent, during running, it is reasonable to suggest that the increase in oxygen uptake is less than what should be expected from the increase in the mass of muscles activated during "ski-walking".

However, to what extent a larger muscle mass as such may explain the observed differences in maximal oxygen uptake is unclear as the values for maximal oxygen uptake during swimming (i.e. arm plus leg exercise) are shown to be lower than the values obtained during bicycling or running (Holmér 1972). Furthermore, there was no difference in oxygen uptake (Åstrand and Saltin 1961b) or cardiac output (Stenberg et al 1967) when leg exercise (bicycling) was compared with arm plus leg exercise (simultaneously cranking and bicycling). It is, however, possible that simultaneously cranking and bicycling or running at high speeds and low grades are technically so difficult that this limits the performance before the maximal oxygen uptake is reached.

With regard to the differences in maximal oxygen uptake between uphill running and bicycling, Glassford et al (1965) suggested that local muscular fatigue in the muscles of the lower extremities during maximal bicycling might limit the work performance, and that this happens before the oxygen transport system is maximally taxed. The results of Hoes et al (1968) which showed that the peak load during a pedal revolution during bicycling is twice as high as the actual load setting may be interpreted to support this view. With regard to the contraction frequencies of the muscles during work, it is also worth noticing the studies of Folkow and co-workers (Folkow et al 1970, Folkow et al 1971) showing that there is almost no inflow of blood during the contraction phase whereas each contraction is accompanied by a large increase in the venous outflow. It was also shown that the blood flow through the muscle and the venous return to the heart was markedly affected by the contraction frequency. Thus, from the results of the present investigation and from those of others, there seems to be an optimal relationship between the force of each muscle contraction and the contraction frequency of the muscles (viz. step frequency during running and pedal frequency during bicycling) and that this is an important factor for increasing the oxygen uptake to the highest possible level.

It is known that the effect of warm-up (Taylor et al 1955) or a hot environment (Rowell et al 1964) are factors which may influence the maximal oxygen uptake. However, in the present investigation all subjects performed almost the same warm-up program and all experiments were performed at approximately the same environmental temperature (i.e. about 20°C). Therefore, it is not likely that these factors have influenced the results of the present investigation.

Although no definite explanation can be given for the above described differences in maximal oxygen uptake during different types of work, there are some practical implications to be noted. To ensure that the highest possible oxygen uptake is reached arm plus leg exercise (i.e. "ski-walking") should be used. However it should be emphasized that the values for maximal oxygen uptake obtained during "ski-walking" and uphill running were almost the same. For the bicycle ergometer test a pedal frequency of 60 to 70 rpm is recommended. Furthermore it should be stressed that the term maximal oxygen uptake refers only to the type of exercise used for the test. A strict comparison of the values for maximal oxygen uptake therefore requires strictly standardized conditions for the determinations.

The question concerning possible limiting factors has been debated for at least 50 years. Hill and Lupton in 1923 stated that it is still an open question whether the oxygen uptake during maximal exercise is limited by the heart or by the lungs. Christensen et al. (1934) listed 5 main factors influencing the oxygen supply to the muscles. These were 1) pulmonary ventilation 2) rate of diffusion of oxygen from alveolar air to the blood 3) cardiac output 4) the rate of blood flow through the muscles, and 5) the oxygen diffusion from the muscle capillaries to the cells. In later studies (Åstrand 1952, Taylor et al. 1955, Mitchell et al. 1958a, Åstrand and Saltin 1961b, Rowell 1962, Saltin 1964, Holmgren 1967, Kajzer 1970, Karlsson 1971, Gollnick et al. 1972 and others) these and other factors have been discussed. The fact that the identification of the possible limiting factors for maximal oxygen uptake is a complex problem to analyze is at least partly illustrated by the fact that the transport of oxygen from air to the mitochondria in the cells includes two convective systems (i.e. ventilation and circulation) and two diffusing systems (i.e. from the alveolar air to the blood and from the capillaries to the mitochondria). On the basis of the results of the present investigation some of the different factors which have been considered to influence the maximal oxygen uptake will be discussed.

The pulmonary ventilation is not considered to be a limiting factor for the maximal oxygen uptake under normal conditions and in young healthy subjects. The results of the present investigation (Fig. 3-5 and Fig. 3-6) may be interpreted as supporting this view. It was shown in Fig. 3-6 that with increasing pedal frequency during maximal bicycle exercise the oxygen uptake levelled off, the pulmonary ventilation however continued to increase. Furthermore, maximal oxygen uptake was higher during uphill running than during bicycling (50 rpm) while the pulmonary ventilation was the same (Fig. 3-5). These results are in agreement with several earlier studies, reaching the same conclusion (Åstrand 1952, Mitchell et al. 1958a, Rowell 1962, Saltin et al. 1968).

The concept that pulmonary ventilation does not represent a limiting factor for the transport of oxygen during maximal exercise is also supported by the following evidence. The pulmonary ventilation normally bears an approximately linear relationship to the oxygen uptake (or work load) up to some critical level representing approximately 60-70 percent of the individual's maximal oxygen uptake. Beyond this critical level the ventilation increases out of proportion to the oxygen consumption (Åsmussen and Nielsen 1946). The increase in pulmonary ventilation is brought about by a progressive increase in both tidal vo-

respiratory rate. The former increases more steeply than the latter during light to moderate loads. The respiratory adjustment to further increase in oxygen uptake however is almost entirely due to an acceleration in the rate of breathing (Christensen 1932). However neither of these factors (i.e. tidal volume or respiratory rate) reach their highest possible values during maximal exercise. According to Drippa and Comroe (1947) and Freedman (1970) healthy subjects utilize less than 70 percent of their maximal voluntary volume during work loads demanding maximal oxygen uptake. On the other hand it should be emphasized that the maximal voluntary volume is defined as the highest possible ventilation during 10-15 seconds. The highest possible ventilation during 4 min is shown to be only 70-75 percent of the maximal voluntary volume (Freedman 1970). The relationship between the pulmonary ventilation and the pulmonary circulation is another important factor. During exercise there is an increase in the overall ventilation/perfusion ratio and the distribution of air and blood to the different parts of the lungs becomes less uneven (West and Dollery 1960). During maximal exercise there is also an increase in oxygen tension and a decrease in carbon dioxide tension in the alveolar air (Asmussen and Nielsen 1958). Thus, according to the above mentioned studies, the pulmonary ventilation seems to be large enough to secure an adequate alveolar oxygen tension even during maximal exercise.

Normally the diffusion of oxygen from the alveolar air to the blood is not considered a limiting factor for maximal oxygen uptake in young subjects (Holmgren and Åstrand 1966, Saltin et al. 1968). This view is based at least partly on evidence from measurements of diffusion capacity (Saltin et al. 1968), arterial oxygen tension (Mitchell et al. 1958) and oxygen saturation of arterial blood (Åstrand and Linderholm 1958, Mitchell et al. 1958, Asmussen and Nielsen 1960, Howell 1964, Saltin et al. 1968) during submaximal and maximal exercise. On the other hand there is evidence which suggests that the diffusing capacity of the lungs might be a limiting factor during maximal exercise in older subjects (Cohn et al. 1954). This finding may at least partly explain the reduction in maximal oxygen uptake with age which was observed both in female and male subjects in the present investigation.

Oxygen uptake at rest and during exercise is determined by the product of cardiac output and the arteriovenous oxygen difference. Thus, if it is considered that neither pulmonary ventilation nor the diffusion of oxygen from the alveolar air to the blood limits the oxygen uptake during maximal exercise two main possibilities seem to exist. First, that the maximal oxygen uptake may be limited by the capacity to transport oxygen from the lungs to the active tissues. This could be due to either a low oxygen carrying capacity of the blood (i.e. a low hemoglobin concentration) or an inability to increase the cardiac output or a combination of both factors. The cardiac output, in turn, could be limited by a low maximal heart rate, a low maximal stroke volume or a combination of both. Secondly that the maximal oxygen uptake may be limited by an inability of the tissues to extract more oxygen from the blood, i.e. a low arteriovenous oxygen difference. This could be due to an inefficient distribution of the cardiac output or a low capacity of the active tissues to consume the oxygen brought to them.

A low oxygen carrying capacity of the blood (i.e. a low hemoglobin concentration) is the first factor to be analyzed. Cross-sectional analysis of the

results from the present investigation showed a close association between hemoglobin concentration and maximal oxygen uptake when the whole material (i.e. school children and students) was considered. However this association could not be confirmed when the correlation analysis was performed for each of the subgroups separately (i.e. schoolgirls, schoolboys, female and male students). Furthermore it was shown (Fig 4-2) that at the same maximal oxygen uptake (for instance 2.5 l/min) the hemoglobin concentration might vary between 11.5 and 15.0 g/100 ml. On the other hand at the same hemoglobin concentration (for instance 13.6 g/100 ml) maximal oxygen uptake might vary between 1.8 and 5.2 l/min. These results show that the same maximal oxygen uptake may be transported with quite different hemoglobin concentrations, and that subjects having the same hemoglobin concentration might have different maximal oxygen uptakes. These observations are in agreement with several previous investigations (Åstrand 1952, von Döbeln 1956, Buskirk and Taylor 1957). It was shown by von Döbeln (1956) that the correlation coefficient between total hemoglobin content and maximal oxygen uptake was 0.725. However the correlation coefficient between fat free body weight and maximal oxygen uptake was even higher (i.e. 0.756). Thus the partial correlation coefficient between total hemoglobin content and maximal oxygen uptake was only 0.246. On the basis of these results, von Döbeln (1956) concluded that the main reason for the close correlation between the total hemoglobin content and the maximal oxygen uptake was that the total hemoglobin content is related to the body size. The results of the present investigation may be interpreted to support the conclusion of von Döbeln (1956).

The only suggestion of an impaired oxygen transport in subjects with low hemoglobin concentration is given by Sproule et al. (1960). These authors observed lower values for maximal oxygen uptake in 9 anemic men (hemoglobin concentration 5.2 - 9.3 g/100 ml) than the corresponding values for healthy men of comparable age studied by Mitchell et al. 1958a, b. Whether such a comparison is relevant or not, might be questionable. There are several points in the study of Sproule et al. (1960) which should not be overlooked. Maximal oxygen uptake was neither obtained in all subjects (i.e. only in 6 of the 9 subjects) nor measured after restoration of the hemoglobin concentration. Since all 9 subjects were included in the study the mean value for maximal oxygen uptake is too low. Furthermore it is interesting to note that the anemic subjects were able to run on the treadmill at the same speed and duration as that of the healthy subjects studied by Mitchell et al. (1958a). The possible explanation that anaerobic processes might have been taxed to a larger extent in the anemic subjects, is not supported by the fact that the blood lactate concentrations were lower in the anemic subjects than in the healthy ones. Finally it should be emphasized that the values for maximal oxygen uptake were not corrected for differences in body size, and that at least 3 of the subjects studied by Sproule et al. (1960) had body weights of 56.2, 55.2 and 49.4 kg. Thus, it appears that the maximal oxygen uptake in the anemic subjects, if expressed in ml/kg x min, might possibly be the same, or almost the same, as that observed in the healthy subjects.

The results discussed above were all obtained in cross-sectional information about the effect of variation in the hemoglobin on the

oxygen uptake in longitudinal studies, on the other hand is limited. The results of the longitudinal experiments in the present investigation showed that in the same subject hemoglobin concentration may increase (or decrease) considerably while maximal oxygen uptake may remain unchanged increase or decrease. In some subjects the hemoglobin concentration remained at the same level while maximal oxygen uptake increased considerably. Thus, it may be concluded that the cross-sectional and longitudinal analysis of the present investigation showed no consistent relationship between hemoglobin concentration and maximal oxygen uptake.

These observations are in agreement with the results of several previous investigations. Saltin (1964) for instance found no change in maximal oxygen uptake after thermal dehydration which increased the hemoglobin concentration by 8 percent and reduced the plasma volume by 14 percent. Robinson et al. (1966) found no change in maximal oxygen uptake after transfusion of one liter of blood. Furthermore Saltin et al. (1968) observed only small changes in the relative or total hemoglobin content after bed rest or training. Maximal oxygen uptake, however varied considerably.

Several investigations have on the other hand showed a reduction in the physical performance capacity accompanying the lowered hemoglobin concentration after blood loss (Karpovich and Millman 1942 Balke et al. 1954 Rowell et al. 1964 Ekblom et al. 1972). However with regard to the effect of the reduced hemoglobin concentration on the capacity to transport oxygen, the various studies have yielded conflicting results. Thus, Rowell et al. (1964) for instance, found that a reduction in hemoglobin concentration of 14 percent due to repeated phlebotomies over an eight-day period gave almost no reduction (4 percent) in maximal oxygen uptake. Ekblom et al. (1972) on the other hand showed that hemoglobin concentration decreased, on the average 13 percent after blood loss of 800 ml in 3 subjects (group I) and 18 percent after a blood loss of 1200 ml in 4 subjects (group II). Maximal oxygen uptake decreased to the same extent, i.e. 13 and 18 percent in group I and group II respectively. In the same experiment it was shown that after reinfusion of red blood cells both hemoglobin concentration and maximal oxygen uptake increased.

Thus, according to the above mentioned studies, variations in the total blood volume, plasma volume or red cell volume do not seem to give any consistent effect on the maximal oxygen uptake. Furthermore, a comparison of the results obtained in these studies, which included transfusion of whole blood (Robinson et al. 1966) or red blood cells (Ekblom et al. 1972) phlebotomies (Rowell 1962 Ekblom et al. 1972) dehydration (Saltin 1964) and bed rest and training (Saltin et al. 1968) might be questionable. All the different experimental conditions, as such, might have effected the physical performance capacity of the subjects and thus indirectly also the transport of oxygen.

If the same amount of oxygen is transported from the lungs to the muscles per unit of time with low and high hemoglobin concentrations, compensatory mechanisms must be involved. The results of the present study as well as several other investigations (Sproule et al. 1960 Murray et al. 1962 Åstrand et al. 1964 Grimby et al. 1966 Crowell and Smith 1967 and others) indicate that variations in the hemoglobin concentration may be compensated by changes in the cardiac output or arteriovenous oxygen difference, or by a combination of both factors.

The results from two measurements on the same well-trained athlete (A.11) performed with an interval of 5 years, indicate that a fall in the hemoglobin concentration may be compensated for by an increase in cardiac output. A.11 (no 12) was studied the first time in 1962 by Åstrand et al (1964) and the second time in 1967 (Eklöf and Hermansen 1968). Maximal oxygen uptake was 5.39 l/min in 1962. This oxygen uptake was achieved by a cardiac output of 28.8 liter/min and an arteriovenous oxygen difference of 187 ml per liter of blood. The hemoglobin concentration was 16.5 g/100 ml. This gives a cardiac output of 5.34 liters per liter of oxygen consumed. When the same subject visited the same laboratory 5 years later (Eklöf and Hermansen 1968) his maximal oxygen uptake was increased slightly to 5.60 l/min. This oxygen uptake was brought about by a cardiac output of 34.4 l/min and an arteriovenous oxygen difference of 163 ml per liter of blood. The hemoglobin concentration on this occasion was 14.7 g/100 ml and the cardiac output per liter of oxygen consumed was 6.1 liters. Other results which add circumstantial support to the concept that variations in the hemoglobin concentration might be compensated for by changing the cardiac output are reported by Grimby et al. (1966). In this study old (45–55 years) still active athletes were found to have an average maximal oxygen uptake of 3.56 l/min. The corresponding value for the cardiac output was 26.8 l/min. Hemoglobin concentration was 13.5 g/100 ml (average value). This gives an average cardiac output of 7.5 liters of blood per liter of oxygen consumed. In the study by Åstrand et al. (1964) in young healthy males the corresponding figure was 5.9 liters blood per liter of oxygen consumed. The mean value for the hemoglobin concentration was 15.6 g/100 ml in these subjects. Female subjects are known to have lower mean values for hemoglobin concentration. Consequently a higher cardiac output per liter of oxygen consumed should be anticipated both during submaximal and maximal work. In fact this was shown by Åstrand et al. (1964). The mean values for hemoglobin concentration and oxygen uptake during maximal work were 13.6 g/100 ml and 2.60 l/min, respectively. The corresponding mean value for cardiac output was 18.4 l/min, representing 7.1 liters of blood per liter of oxygen consumed. In another group of subjects with fairly low hemoglobin values, the pubertal boys studied by Eriksson (1972) a cardiac output of 6.9 liters per liter of oxygen consumed during maximal exercise was observed. In the anemic subjects studied by Spronkle et al. (1960) the corresponding value was 12.8 liters of blood per liter of oxygen consumed. Thus, there seems to be considerable evidence which suggests that variations in the hemoglobin concentration could be compensated for by changing the cardiac output.

Another consideration that adds support to the concept of compensatory mechanisms for the capacity to transport oxygen in relationship to variations in the hemoglobin concentration is the series of studies showing an important relationship between the metabolism of the red blood cells and its function as an organ for oxygen transport (Chanutlin and Cornish 1967, Benesch and Benesch 1967, de Verdier et al. 1969, Brewer and Eaton 1971). It was shown by Chanutlin and Cornish (1967) and Benesch and Benesch (1967) that the presence of phosphorylated intermediates, 2,3-diphosphoglycerate (DPG) or adenosine triphosphate (ATP) caused a concomitant reduction in the oxygen affinity of hemoglobin solutions. Eaton and Brewer (1968), Eaton et al. (1969, 1970) showed a signif

negative correlation between red blood cells DPG and the hemoglobin concentration. Experimentally produced polycythemia in rats is shown to cause low levels of DPG (Gerlach et al 1970) while in anemia of various etiologies the level of DPG is high (Hjelm 1969 Eaton et al 1970). The results of these studies indicate that for instance a lowering of the hemoglobin concentration might cause changes in the red blood cells by which more oxygen is released from the hemoglobin at a certain oxygen tension in the tissue. Furthermore it is also shown that the rate of desaturation may be significantly increased in anemic subjects (Brewer and Easton 1971). That these mechanisms probably play an important role for the oxygen transport during maximal exercise in subjects having low hemoglobin concentrations is indicated by the low oxygen content of femoral venous blood (i.e. 1.35 vol %) in the anemic subjects studied by Sproule et al (1960) compared with the corresponding value in normal subjects, i.e. 6.88 vol.% (Mitchell et al 1958a).

Although the results of the present study and those of many others indicate that variations in the hemoglobin concentration may be effectively compensated by changing the cardiac output and/or arteriovenous oxygen difference studies with physical models (Crowell and Smith 1967) and in dogs (Murray et al. 1962) have suggested that there seems to be an optimal hematocrit where the oxygen transport is maximal. The studies of Murray et al. (1962) also indicated that the hemodynamic adjustments in normovolemic anemia and polycythemia were insufficient to maintain normal oxygen delivery.

The capacity of the heart to increase the cardiac output is, maybe the factor most frequently considered as the main limiting factor (Mitchell et al 1958a, Åstrand and Saltin 1961b, Rowell 1962, Bevegård and Shepherd 1967, Ouellet et al 1969, Gollnick et al. 1972, Miyamura and Honda 1972 and others). The view that cardiac output is an essential factor for the magnitude of the maximal oxygen uptake, is supported by evidence from studies on patients with pure mitral stenosis (Blackmon et al. 1967), sedentary healthy subjects (Åstrand et al 1964) and endurance athletes (Ekblom and Hermansen 1968). These studies showed that subjects with low maximal oxygen uptake had low cardiac outputs while subjects with high maximal oxygen uptakes were characterized by high cardiac outputs.

The cardiac output is determined by the product of heart rate and stroke volume. Accordingly cardiac output may be limited by an inability to increase the heart rate to that of the stroke volume. With regard to the first possibility it is worth noticing that the maximal heart rate is found to be approximately the same (190-200 beats/min) in subjects with low average and high maximal cardiac outputs (Åstrand et al. 1964, Blackmon et al. 1967, Ekblom and Hermansen 1968). Physical training increases both maximal oxygen uptake and maximal cardiac output (Rowell 1962, Saltin et al. 1968, Ekblom 1969). Maximal heart rate, however, remains almost unchanged. There are even studies indicating a lowering of the maximal heart rate in connection with training (Andersen 1967a, Davies 1967).

Changes in the stroke volume seem to be the most important factor by which cardiac output is altered in subjects with "normal" hemoglobin values (Åstrand et al. 1964, Blackmon et al. 1967, Ekblom and Hermansen 1968). This was also the case in the present investigation where a 6 percent higher cardiac output was observed during uphill running than during bicycling. This increase in cardiac output was brought about by only a small (approximately 1 percent) increase in

maximal heart rate, while increased stroke volume contributed approximately 5 percent. Altered stroke volume seems also to be an important factor with regard to the observed changes in cardiac output with variations in the hemoglobin concentration (Ekblom and Hermansen 1968). Although the mechanisms by which the stroke volume is altered are unknown, results from animal studies and investigations on human subjects seem to indicate that cardiac and extracardiac factors are involved.

With regard to the changes in stroke volume (i.e. cardiac output) in relation to variations in the hemoglobin in the hemoglobin concentration (i.e. hematocrit) and to exercise (or a combination of both) the present study and other investigations (Murray et al. 1962, Fowler and Holmes 1971) seem to indicate that changes in the peripheral resistance may be one important factor. The mechanisms by which the peripheral resistance is varied in the intact organisms are unclear but variations in the viscosity of the blood are one essential factor (Murray et al. 1962, Crowell and Smith 1967, Agarwal et al. 1970). However as indicated by Escobar et al. (1966) and Fowler and Holmes (1971) improved ventricular function is another possibility. The reflex control of the veins is another important factor with special reference to the changes during exercise (Bevegård and Shepherd 1965, Shepherd 1966). However if or to what extent the reflex control of the veins is influenced by the hematocrit is not known.

Although considerable evidence indicates that the maximal oxygen uptake is limited by the capacity of the heart to transport oxygen (i.e. blood) to the working muscles, several investigators have suggested that maximal oxygen uptake is limited by peripheral factors. Åstrand (1952) for instance suggested that the capacity of the heart to increase the cardiac output is greater than the ability of the muscles to receive it. Thus, it was indicated that the limiting factor might be found in the capacity of the vascular bed of the muscles.

In the present study the capillary density was found to be approximately 600 capillaries per mm^2 both in the well-trained and untrained subjects. The maximal oxygen uptake, however was 71.4 $\text{ml/kg} \times \text{min}$ in the well-trained and 50.2 $\text{ml/kg} \times \text{min}$ in the untrained subjects. Thus, the well-trained subjects were able to transport approximately 20 ml oxygen per kilogram body weight and minute more than the untrained subjects. Although there might be a difference in the lean body mass between the well-trained and untrained subjects it still means that a larger amount of oxygen is transported per unit muscle weight and time by the well-trained subjects. Thus, it appears that the number of capillaries per mm^2 as such does not represent a limiting factor for the transport of oxygen during maximal exercise in man. However it was also shown in the present investigation that the average size of the muscle cells was larger in the well-trained as compared to the untrained subjects. Consequently each muscle cell was supplied with a larger number of capillaries in subjects with high maximal oxygen uptake compared with subjects with lower maximal oxygen uptake. However when the diffusion distance was calculated according to the method of Krogh (1919) no statistically significant differences between the two groups of subjects could be observed. Thus, according to the results of the present study it appears that the diffusion distance as such, probably does not limit the oxygen transport in these subjects.

Mathematical models describing the oxygen transport in peripheral tissues 1

negative correlation between red blood cells DPG and the hemoglobin concentration. Experimentally produced polycythemia in rats is shown to cause low H_2O DPG (Gerlach et al. 1970) while in anemia of various etiologies the level of high (Hjelm 1969 Eaton et al. 1970). The results of these studies indicate the instance a lowering of the hemoglobin concentration might cause changes in red blood cells by which more oxygen is released from the hemoglobin at a low oxygen tension in the tissue. Furthermore it is also shown that the H_2O desaturation may be significantly increased in anemic subjects (Brewer and I. 1971). That these mechanisms probably play an important role for the O_2 transport during maximal exercise in subjects having low hemoglobin concentrations is indicated by the low oxygen content of femoral venous blood (i.e. vol %) in the anemic subjects studied by Sproule et al. (1960) compared with corresponding value in normal subjects, i.e. 6.88 vol.% (Mitchell et al. 1955).

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proposed by Staunby and Otis (1964). However, whether or not the oxygen tension of the venous blood is representative for the oxygen tension in all capillaries is unknown. It is, for instance, unlikely that all motor units are activated at the work intensity necessary to obtain the maximal oxygen uptake. Consequently, the venous blood draining the muscle will probably be a mixture of blood with low and higher oxygen tensions. Furthermore, Forster (1967) has indicated that there are differences in the oxygen tensions, not only between the venous blood and the cells, but also within the muscle cell itself. There is also evidence which suggests that there might be a small oxygen difference in the oxygen tension even within the mitochondria (Forster 1967). If this is correct, it seems difficult to assess the oxygen tension in the cells from measurements of oxygen tension in the venous blood from the tissue. Thus, a high oxygen tension in the venous blood does not necessarily mean that the oxidative capacity of the muscles represents the limiting factor.

With regard to the arteriovenous oxygen difference during maximal exercise, surprisingly small differences have been observed between groups of subjects with large differences in maximal oxygen uptake. Thus, the arteriovenous oxygen difference was found to be approximately the same in patients with mitral stenosis (Blackmon et al. 1967), sedentary healthy subjects (Åstrand et al. 1964) and athletes (Rowell 1962, Ekblom and Hermansen 1968). On the other hand, it was shown by Rowell (1962) and Saltin et al. (1968) that the arteriovenous oxygen difference was increased after training. Holloszy and co-workers (Holloszy 1967, Holloszy et al. 1971) have shown that there is an increase in the mitochondrial enzymes during physical training. The higher oxygen uptake obtained after training would, according to Holloszy et al. (1971), be explained by the higher capacity to oxidize pyruvate. However, in the results of Gollnick et al. (1972) it was indicated that the oxidative capacity of the skeletal muscles of both trained and untrained subjects exceeded the values observed for maximal oxygen uptake.

Thus, although the present investigation has provided evidence which may be interpreted to support the view that maximal oxygen uptake is limited by the capacity of the circulatory system to transport oxygen to the muscles, there are still several questions yet to be answered.

been worked out by Krogh (1919) Fenn (1927) Hill (1928) Roughton (1952) Kety (1957) Hill (1965) and Thews (1968). Although there seems to be general agreement about the diffusion theory, necessary experimental evidence to verify the calculations in the different models has been difficult to obtain. According to the above cited studies, a high number of capillaries would facilitate the oxygen transport in the muscles. However, the result of the present investigation showed that the well-trained subjects were able to transport more oxygen per unit muscle mass and time than the untrained subjects, the diffusion distance being the same. However, with regard to the oxygen diffusion in skeletal muscles, the capillary density is probably only one of the many important factors. The presence of myoglobin is another important factor. It was shown by Scholander and Hemmingsen (Scholander 1960, Hemmingsen and Scholander 1960, Hemmingsen 1963, Hemmingsen 1965) that the presence of hemoglobin or myoglobin in a solution may facilitate the oxygen diffusion. It has also been suggested that the presence of myoglobin in the skeletal muscle may enhance the oxygen diffusion and increase the oxygen supply to a significant extent. According to the calculations of Wittenberg (1965) the flux of oxygen facilitated by myoglobin is about 6 times greater than the ordinary diffusive flux. In other words, approximately 80 percent of the oxygen concerned reaches the mitochondria by myoglobin facilitated diffusion. It was shown by Scholander (1960) that the oxygen flux increased with increasing concentration of the pigment (myoglobin or hemoglobin). As indicated by the studies of Whipple (1926) on dogs, and by Pettengale and Holloszy (1967) on rats, physical training may increase the concentration of myoglobin in skeletal muscle. The results of Gollnick et al (1972) might indicate that this also happens in human skeletal muscle during training. Thus, if this concept is correct, the higher oxygen uptake observed in the well-trained subjects of the present investigation may be at least partly explained by a higher concentration of myoglobin in the muscles.

The ability of the active tissues to extract oxygen delivered by the cardiovascular system is another possible limiting factor. If this was the case, a low arteriovenous oxygen difference should be expected. A low arteriovenous oxygen difference could be due to an inefficient distribution of the cardiac output. In fact, this was observed in patients with vasoregulatory asthenia (Holmgren et al 1957). A low arteriovenous oxygen difference could however also be due to a metabolic deficiency, i.e. an inability of the mitochondria to oxidize pyruvate. This possibility is supported by the results of Kaijser (1970) who reported only moderately reduced blood lactate concentration in deep venous blood although the arterial oxygen tension was increased significantly. The author concluded that the links in the oxygen transport system must be fully saturated with oxygen and that the limiting factor for the oxygen consumption must be found at the mitochondrial level. Results from measurements of the oxygen tension in the venous blood draining the muscles during maximal exercise (Keul et al 1967, Doll et al 1968, Kaijser 1970, Pirnay et al 1972) are also interpreted to support the view that oxygen consumption is limited by the capacity of the muscles to consume the oxygen brought to them. In all these studies, the oxygen tension in the venous blood was found to be higher than the critical oxygen tension, (i.e. the oxygen tension below which the rate of oxygen uptake by the muscles is unaffected) of 10 mm Hg as

increased markedly while maximal oxygen uptake remained at the same level. It is emphasized, however, that severely anemic subjects were not included in the study and further examinations of such subjects are necessary to ascertain the limit below which anemia causes measurable impairment of the capacity to transport oxygen.

Capillary density in muscle samples obtained by needle biopsies from the lateral portion of the muscle quadriceps femoris was measured using standard histological techniques and the PAS-staining reaction in 8 untrained students and 7 well trained endurance athletes. Mean values (\pm SE) of the capillary density were found to be 611 ± 34.3 and 599 ± 26.6 for the well trained and the untrained subjects, respectively. Statistical analysis revealed no significant difference between the two groups of subjects. The average size of the muscle fibres in the well-trained muscle was found to be significantly larger than in the untrained muscle. Consequently the number of capillaries per muscle fibre was higher in the well-trained subjects than in the untrained ones. Calculations of the diffusion distance (i.e. average half-distance between two capillaries) in the well-trained and untrained muscle, revealed no statistically significant difference.

The variations in maximal oxygen uptake in relation to sex, age and level of physical activity in different groups of the Norwegian population were investigated. Altogether 593 female and male subjects between 11 and 59 years of age were studied. The results obtained in the different groups of the Norwegian population are compared with similar results from other countries.

On the whole, the mean values for maximal oxygen uptake in the Norwegian subjects were higher than in all other studies reported in the literature.

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Appendix

Table 1. Mean values for oxygen uptake, pulmonary ventilation, heart rate, blood lactate concentration and work time during maximal bicycle and treadmill exercise for groups A through G.

Subjects	Groups	No.	Oxygen uptake l/min STPD		Pulmonary ventilation l/min BTPS		Heart rate beats/min		Blood lactate concentration mM		Work time minutes	
			Bicycle	Treadmill	Bicycle	Treadmill	Bicycle	Treadmill	Bicycle	Treadmill	Bicycle	Treadmill
A	8		4.63	5.04	181.8	161.3	188.1	192.3	13.0	13.4	5.8	8.6
			$p < 0.001$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$	
B	5		3.95	4.29	137.7	139.6	193.8	200.4	13.9	16.4	5.9	4.7
			$p < 0.001$		$p > 0.05$		$p < 0.05$		$p > 0.05$		$p < 0.05$	
C	6		3.17	3.54	130.2	132.9	196.0	197.8	16.4	17.6	4.7	4.6
			$p < 0.001$		$p > 0.05$		$p < 0.05$		$p > 0.05$		$p > 0.05$	
D	5		4.34	4.65	136.9	149.3	187.0	187.0	14.5	13.3	5.2	5.2
			$p < 0.001$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$	
E	10		4.35	4.75	136.5	134.5	178.6	179.7	13.4	14.3	4.4	3.0
			$p < 0.01$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$	
F	14		4.44	3.68	118.0	114.6	177.6	176.0	10.3	11.3	4.4	4.6
			$p < 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$	
G	7		3.36	3.38	120.7	117.9	186.1	185.6	13.8	12.9	3.0	4.3
			$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p > 0.05$	

Table 2. Individual and mean values \pm standard error of the mean and standard deviation for oxygen uptake, cardiac output, heart rate, stroke volume, arterial pressure, mean arterial pressure and arterial oxygen content during maximal bicycle and treadmill exercise

Subjects	Oxygen uptake l/min		Cardiac output l/min		Heart rate beats/min		Stroke volume ml		(A-V) O_2 -diff $\text{vol.}\%$		Mean art. press. mm Hg		Art. O_2 content $\text{vol.}\%$	
	bicycle	treadmill	bicycle	treadmill	bicycle	treadmill	bicycle	treadmill	bicycle	treadmill	bicycle	treadmill	bicycle	treadmill
Q.E.	3.91	4.58	23.2	27.3	180	193	129	141	16.8	18.0	121	109	19.2	19.1
L.L.	4.66	4.91	24.7	26.6	187	187	132	141	18.9	18.6	110	97	21.2	20.7
J.H.	3.32	3.48	21.0	20.0	190	193	110	104	15.8	17.3	101	106	19.0	19.4
K.L.	3.44	3.53	19.2	19.3	188	190	102	102	17.9	18.3	126	104	19.9	20.5
O.S.	3.92	4.29	26.2	26.7	193	193	136	139	18.0	16.0	94	81	17.7	17.6
O.S.	4.34	4.81	27.8	28.3	190	193	143	146	16.5	16.3	115	102	17.8	18.0
T.O.	4.39	4.58	27.7	28.8	185	191	150	151	16.6	15.9	152	136	18.5	17.9
T.T.	4.73	4.84	28.8	29.4	179	184	148	160	17.8	18.9	130	114	17.6	17.1
L.H.	4.33	4.74	25.6	28.0	192	196	133	148	16.5	16.3	131	104	18.9	18.3
P.S.	4.99	5.12	27.5	28.3	172	173	158	163	16.5	16.1	-	-	19.8	18.8
P.O.R.	4.21	4.66	26.3	27.8	179	178	147	158	14.7	16.8	118	140	18.9	19.0
H.N.	4.52	4.70	26.3	28.0	189	183	136	133	16.1	16.8	138	150	19.8	19.1
C.R.	5.21	5.77	35.4	37.8	183	188	193	201	14.7	15.3	127	120	18.6	17.8
Mean	4.31	4.57	26.9	27.5	183.2	187.9	139.9	146.2	16.6	16.7	121.9	110.3	19.0	18.7
SE	0.16	0.17	1.1	1.3	1.8	1.9	6.4	7.2	0.4	0.3	4.3	4.5	0.3	0.3
SD	0.36	0.60	3.8	4.5	6.2	6.6	22.3	24.9	1.4	1.0	15.9	14.9	1.0	1.1
	$p < 0.001$		$p < 0.001$		$p < 0.01$		$p < 0.01$		$p > 0.6$		$p < 0.001$		$p < 0.01$	

Table 3. Correlations () between hemoglobin concentration and maximal oxygen uptake in the total material and in the various subgroups of students and school children.

Material	Maximal oxygen uptake			
	1st examination		2nd examination	
	l/min	ml/kg min	l/min	ml/kg x min
Total material (Students and school children)	+0.67 $p < 0.001$ ($n = 109$)	+0.07 not signif. ($n = 109$)	+0.59 $p < 0.001$ ($n = 208$)	+0.24 $p < 0.001$ ($n = 208$)
Male students	-0.10 not signif. ($n = 20$)	+0.02 not signif. ($n = 20$)	-0.30 not signif. ($n = 16$)	-0.50 not signif. ($n = 16$)
Female students	-0.25 not signif. ($n = 21$)	+0.22 not signif. ($n = 21$)	-0.15 not signif. ($n = 15$)	+0.15 not signif. ($n = 15$)
Schoolboys	+0.30 not signif. ($n = 37$)	+0.07 not signif. ($n = 37$)	+0.58 $p < 0.001$ ($n = 93$)	+0.15 not signif. ($n = 93$)
Schoolgirls	+0.55 $0.01 < p < 0.05$ ($n = 31$)	-0.55 not signif. ($n = 31$)	+0.05 not signif. ($n = 34$)	+0.07 not signif. ($n = 34$)

Table 4. Anthropometric and physiological data from the cross-sectional studies / school children. Mean values \pm standard error of the mean and standard deviation in the different age groups are given.

Sex	Number of subjects	Age years	Height cm	Weight kg	Oxygen uptake		Heart rate beats/min	Pulmonary ventilation l/min	Blood lactate concentration mmol
					l/min	ml/kg min			
Female	15	11.0 \pm 0.1	142.1 \pm 1.9	34.9 \pm 1.5	1.84 \pm 0.09	55.0 \pm 1.0	208.3 \pm 2.1	60.5 \pm 3.7	8.5 \pm 0.5
		0.4	7.0	8.6	0.31	3.8	7.9	13.9	2.0
Male	21	11.0 \pm 0.1	140.4 \pm 1.9	32.4 \pm 2.8	2.02 \pm 0.08	59.0 \pm 1.5	202.7 \pm 1.5	67.9 \pm 2.8	7.5 \pm 0.5
		0.4	8.7	12.4	0.57	6.7	6.8	12.6	2.3
Female	38	12.0 \pm 0.1	147.9 \pm 1.0	40.0 \pm 1.0	2.13 \pm 0.05	53.7 \pm 0.6	204.7 \pm 1.0	72.5 \pm 1.7	10.8 \pm 0.5
		0.3	7.8	7.5	0.54	4.6	7.6	12.7	3.3
Male	61	12.0 \pm 0.1	146.7 \pm 0.8	38.1 \pm 0.8	2.25 \pm 0.04	59.4 \pm 0.7	204.7 \pm 1.0	70.5 \pm 1.5	9.7 \pm 0.5
		0.3	6.1	6.1	0.31	5.1	7.9	11.7	2.2
Female	52	13.1 \pm 0.1	155.1 \pm 1.2	45.3 \pm 1.3	2.35 \pm 0.05	52.3 \pm 0.9	204.0 \pm 1.3	83.6 \pm 1.9	11.6 \pm 0.5
		0.4	6.5	7.4	0.39	4.9	7.1	10.3	2.3
Male	25	13.0 \pm 0.1	151.4 \pm 1.8	40.3 \pm 1.2	2.32 \pm 0.08	63.4 \pm 1.3	204.0 \pm 1.3	79.5 \pm 3.1	9.6 \pm 0.4
		0.3	8.5	6.0	0.41	6.1	6.3	13.3	2.0
Female	17	13.9 \pm 0.1	159.1 \pm 0.9	52.6 \pm 1.77	2.61 \pm 0.07	49.8 \pm 1.7	208.4 \pm 2.0	89.5 \pm 2.9	11.2 \pm 0.9
		0.3	3.7	6.5	0.38	6.6	7.7	11.3	3.3
Male	24	13.9 \pm 0.1	159.1 \pm 1.5	48.8 \pm 1.7	3.09 \pm 0.12	63.2 \pm 0.9	203.5 \pm 1.5	102.9 \pm 4.4	11.5 \pm 0.5
		0.3	8.8	8.1	0.58	4.1	7.0	21.5	2.6
Female	12	15.0 \pm 0.1	158.3 \pm 1.5	51.5 \pm 1.8	2.57 \pm 0.11	50.2 \pm 2.1	205.2 \pm 2.7	90.6 \pm 4.3	11.3 \pm 0.9
		0.3	5.0	5.0	0.35	7.0	9.1	14.4	3.8
Male	22	15.0 \pm 0.1	164.6 \pm 1.7	52.7 \pm 1.7	3.27 \pm 0.12	62.1 \pm 1.1	201.8 \pm 1.7	110.7 \pm 4.0	13.0 \pm 0.5
		0.3	7.8	7.7	0.54	5.1	7.9	18.5	2.1
Female	9	16.0 \pm 0.1	164.8 \pm 3.5	55.0 \pm 3.6	2.62 \pm 0.14	47.3 \pm 0.7	199.8 \pm 4.0	88.6 \pm 3.1	11.8 \pm 1.2
		3.3	9.8	9.0	0.38	1.9	11.4	8.9	3.4
Male	13	15.8 \pm 0.1	169.9 \pm 2.2	60.6 \pm 2.0	3.63 \pm 0.15	65.4 \pm 1.3	202.7 \pm 1.9	124.4 \pm 3.2	13.6 \pm 0.8
		0.3	7.5	6.8	0.52	4.6	6.3	11.2	2.9

Maximal values

Table 5. Anthropometric and physiological measurements obtained studies of school children in Class 4. Mean values \pm standard error / the mean and standard deviation for the 6 examinations are given.

Maximal values											
Examination	Sex	Number of subjects	Age, years	Height, cm	Weight, kg	Oxygen uptake			Heart rate, beats/min	Pulmonary ventilation, l/min	Blood lactate concentration, mM
						l/min	ml/kg min	ml/min			
1st	Female	15	12.8 ± 0.1 0.3	152.5 ± 1.7 5.8	44.1 ± 1.8 6.4	2.22 ± 0.07 0.33	80.8 ± 1.2 4.7	205.8 ± 2.9 18.2	73.6 ± 2.3 7.6	11.4 ± 0.6 1.9	
	Male	16	12.7 ± 0.1 0.2	150.1 ± 1.6 6.1	40.3 ± 1.3 5.0	2.35 ± 0.08 0.29	88.4 ± 0.9 8.3	197.3 ± 2.6 18.0	64.7 ± 1.9 7.4	8.0 ± 0.6 2.4	
2nd	Female	15	13.4 ± 0.1 0.3	153.8 ± 1.8 5.5	47.5 ± 2.4 8.2	2.42 ± 0.08 0.37	91.5 ± 1.5 4.6	205.1 ± 2.5 7.8	84.4 ± 3.6 12.5	12.2 ± 0.7 2.3	
	Male	16	13.3 ± 0.1 0.2	153.9 ± 1.8 6.3	43.3 ± 1.4 5.2	2.39 ± 0.08 0.32	66.1 ± 1.1 4.3	200.6 ± 2.2 8.4	84.7 ± 3.6 11.8	12.3 ± 0.7 2.8	
3rd	Female	15	13.8 ± 0.1 0.3	157.2 ± 1.7 6.0	48.9 ± 2.3 7.8	2.56 ± 0.06 0.21	92.0 ± 1.3 4.6	207.8 ± 1.8 8.4	88.8 ± 3.2 11.8	11.9 ± 0.7 2.3	
	Male	16	13.7 ± 0.1 0.2	156.5 ± 1.7 6.7	45.7 ± 1.8 5.8	2.81 ± 0.10 0.40	63.7 ± 1.4 3.5	203.6 ± 2.4 9.5	93.5 ± 3.5 12.8	11.0 ± 0.8 1.9	
4th	Female	15	14.2 ± 0.1 0.3	158.8 ± 1.8 5.8	51.2 ± 2.6 7.1	2.69 ± 0.07 0.23	60.9 ± 1.2 4.3	209.2 ± 2.4 8.2	95.8 ± 3.2 11.0	12.8 ± 0.4 1.5	
	Male	16	14.2 ± 0.1 0.2	161.4 ± 1.9 7.5	48.2 ± 1.7 6.4	3.05 ± 0.12 0.43	63.3 ± 1.3 3.1	201.8 ± 2.0 7.7	98.3 ± 7.7 30.6	10.9 ± 0.3 1.3	
5th	Female	15	14.7 ± 0.1 0.3	159.7 ± 1.8 6.1	52.8 ± 1.9 6.1	2.55 ± 0.07 0.23	48.8 ± 1.0 3.5	206.6 ± 2.2 7.3	89.6 ± 2.8 9.7	12.1 ± 0.6 2.2	
	Male	16	14.7 ± 0.1 0.2	165.0 ± 1.9 7.4	52.2 ± 1.8 7.0	3.13 ± 0.11 0.43	66.1 ± 1.5 3.8	200.7 ± 2.2 8.5	104.5 ± 3.8 14.6	11.3 ± 0.6 2.3	
6th	Female	15	15.2 ± 0.1 0.3	160.7 ± 1.7 6.0	53.1 ± 1.9 6.6	2.46 ± 0.07 0.23	44.6 ± 0.9 3.1	204.9 ± 1.8 8.8	89.8 ± 3.2 11.2	12.0 ± 0.7 2.4	
	Male	16	15.2 ± 0.1 0.2	168.4 ± 1.8 7.1	54.6 ± 1.9 7.4	3.35 ± 0.12 0.48	61.4 ± 1.2 4.7	201.4 ± 1.9 7.5	110.0 ± 4.6 17.9	10.8 ± 0.6 2.4	

Table 6. Anthropometric and physiological data from the longitudinal studies of school children in Class B. Mean values, standard error of the mean and standard deviations for the 6 measurements are given.

		Maximal values									
Examination Date	Sex	Number of subjects	Age years	Height cm	Weight kg	Oxygen uptake			Heart rate beats/min	Pulmonary ventilation l/min	Blood lactate concentration mM
						l/min	ml/kg min	ml/min			
1st	Female	18	11.8 ± 0.1 0.3	145.7 ± 1.7 6.5	38.2 ± 1.5 3.7	2.02 ± 0.07 0.26	33.1 ± 1.1 4.1	204.8 ± 1.7 6.4	66.3 ± 2.3 6.4	11.2 ± 0.4 1.4	
	Male	14	12.0 ± 0.1 0.3	145.4 ± 1.6 3.8	37.4 ± 2.0 7.1	2.10 ± 0.06 0.31	38.1 ± 2.0 7.2	206.4 ± 1.6 5.9	64.0 ± 2.5 8.5	8.7 ± 0.4 1.5	
	Female	18	12.3 ± 0.1 0.3	149.5 ± 1.7 6.4	41.8 ± 1.7 6.4	2.24 ± 0.07 0.35	34.2 ± 1.4 3.3	205.6 ± 2.0 7.5	80.5 ± 2.4 9.0	12.4 ± 0.6 2.1	
2nd	Male	16	12.5 ± 0.1 0.3	148.8 ± 1.8 6.5	39.5 ± 2.0 7.1	2.59 ± 0.07 0.37	61.4 ± 1.8 6.3	206.9 ± 1.6 5.6	74.1 ± 3.1 12.5	11.8 ± 0.5 1.9	
	Female	18	12.8 ± 0.1 0.3	152.1 ± 1.7 6.2	44.8 ± 1.7 6.4	2.42 ± 0.07 0.37	54.7 ± 1.5 5.4	208.5 ± 1.8 6.7	86.5 ± 2.9 10.9	11.8 ± 0.5 2.0	
3rd	Male	14	13.0 ± 0.1 0.3	151.2 ± 2.1 7.7	41.8 ± 2.1 7.7	2.67 ± 0.10 0.36	64.6 ± 1.7 6.0	210.6 ± 1.6 5.6	85.0 ± 3.7 13.2	12.0 ± 0.6 2.3	
	Female	18	13.3 ± 0.1 0.3	155.3 ± 1.6 6.0	44.9 ± 1.6 6.0	2.41 ± 0.06 0.21	51.8 ± 1.5 5.0	203.8 ± 2.4 8.9	89.6 ± 2.5 9.5	12.5 ± 0.5 1.8	
4th	Male	14	13.8 ± 0.1 0.3	163.7 ± 2.4 8.6	45.3 ± 2.2 8.3	2.69 ± 0.12 0.42	62.5 ± 1.2 6.4	206.6 ± 2.0 7.2	91.8 ± 5.6 15.3	11.9 ± 0.5 1.8	
	Female	18	13.8 ± 0.1 0.3	167.9 ± 1.8 5.6	50.1 ± 1.6 8.3	2.48 ± 0.04 0.17	48.8 ± 1.2 4.5	204.7 ± 2.2 8.3	85.8 ± 2.4 8.9	11.0 ± 0.7 2.7	
5th	Male	14	14.0 ± 0.1 0.3	168.1 ± 2.6 9.3	48.1 ± 2.5 8.9	2.99 ± 0.14 0.51	67.6 ± 1.6 8.7	206.3 ± 1.5 5.4	97.4 ± 3.9 21.4	11.4 ± 0.3 1.1	
	Female	18	14.3 ± 0.1 0.3	168.5 ± 1.8 6.5	50.8 ± 1.4 8.3	2.50 ± 0.01 0.17	45.6 ± 1.5 6.7	203.9 ± 1.7 6.3	98.4 ± 2.8 9.3	10.7 ± 0.6 2.3	
6th	Male	14	14.3 ± 0.1 0.3	183.4 ± 2.8 8.1	49.8 ± 2.6 9.2	3.09 ± 0.16 0.56	62.5 ± 1.5 5.8	203.1 ± 1.4 5.2	107.2 ± 6.1 22.1	10.8 ± 0.6 2.2	

Table 7 Anthropometric and physiological data describing the nitroimidazole subjects. Mean values \pm standard error of the mean and standard deviation of the different age group are given.

Measured values										
Sex	Number of subjects	Age group	Height		Weight kg	Oxygen uptake		Heart rate beats/min	Pulmonary ventilation l/min	Blood lactate concentration mmol
			cm	in		l/min	ml/kg min			
Female	14	20-29	169.4 ± 1.3 4.6		60.8 ± 1.8 6.6	2.41 ± 0.07 0.27	37.9 ± 0.8 7.8	205.6 ± 2.6 9.5	81.6 ± 2.0 8.0	11.5 ± 0.5 1.7
Male	20	20-29	181.8 ± 1.3 8.8		73.8 ± 1.0 8.7	3.96 ± 0.11 0.47	54.1 ± 1.5 6.7	201.5 ± 2.0 8.8	149.8 ± 3.9 16.9	12.8 ± 0.6 2.4
Female	8	30-39	165.4 ± 2.7 7.2		63.3 ± 3.0 8.0	2.09 ± 0.09 0.15	33.2 ± 1.5 3.9	185.3 ± 2.3 6.0	60.0 ± 3.8 10.0	-
Male	24	30-39	178.2 ± 1.2 5.6		76.1 ± 1.7 8.1	3.16 ± 0.08 0.39	41.8 ± 1.1 5.0	189.9 ± 1.8 8.8	90.0 ± 3.7 17.6	-
Female	14	40-49	163.2 ± 1.7 6.2		59.8 ± 2.1 7.4	1.95 ± 0.07 0.26	32.9 ± 1.3 4.5	178.4 ± 1.5 9.0	56.7 ± 2.7 9.7	-
Male	16	40-49	179.0 ± 1.4 5.5		75.1 ± 2.5 9.5	2.93 ± 0.09 0.34	39.3 ± 1.0 4.0	188.8 ± 3.4 13.2	83.5 ± 4.0 15.4	-
Female	8	50-59	163.9 ± 1.9 5.1		64.3 ± 4.4 11.7	1.81 ± 0.10 0.27	28.5 ± 1.8 4.7	171.8 ± 4.7 12.5	60.7 ± 5.1 13.5	-
Male	17	50-59	177.1 ± 2.0 7.9		72.1 ± 3.1 12.3	2.58 ± 0.10 0.41	56.1 ± 1.1 4.3	177.1 ± 3.9 15.7	76.2 ± 3.6 14.4	-

Table 2. Pertinent data describing the female physical education students and the different groups of female skiers.

Sports event	Number of subjects	Age years	Height cm	Weight kg	Oxygen uptake			Maximal values		Pulmonary ventilation l/min	Blood lactat concentration mmol
					l/min	ml/kg x min	Heart rate beats/min				
Orientierung	6	23.3 ± 2.4 6.4	160.8 ± 1.7 3.8	60.0 ± 1.9 4.3	3.65 ± 0.3 0.66	60.7 ± 3.3 7.4	184.2 ± 3.0 11.2			113.1 ± 10.1 22.5	12.5 ± 3 3.2
Running 400-1500 m	5	21.4 ± 2.0 4.0	170.2 ± 0.7 1.5	54.6 ± 1.8 3.8	3.36 ± 0.14 0.29	57.3 ± 1.5 3.1	197.6 ± 4.5 9.0			102.5 ± 4.5 9.0	10.8 ± 0.7 1.1
Skiing cross-country	9	23.2 ± 1.8 5.5	163.7 ± 2.1 6.4	60.8 ± 1.4 4.2	3.44 ± 0.08 0.23	56.9 ± 1.0 2.9	191.1 ± 2.8 7.8			106.4 ± 3.8 11.4	11.1 ± 1.2 3.4
Speed skating	8	20.4 ± 1.6 4.1	168.1 ± 1.8 4.8	63.4 ± 2.0 5.2	3.36 ± 0.07 0.18	52.0 ± 1.6 4.2	201.6 ± 3.6 9.5			101.2 ± 3.2 8.5	10.8 ± 0.9 2.3
Gymnastics	11	13.5 ± 0.5 1.6	159.7 ± 1.9 6.1	48.8 ± 2.1 6.5	2.42 ± 0.09 0.28	49.8 ± 1.1 3.5	195.6 ± 2.0 6.4			87.3 ± 4.3 14.1	15.5 ± 0.5 1.6
Physical education students	21	22.2 ± 0.4 1.7	169.6 ± 1.5 6.9	59.3 ± 1.7 7.4	2.71 ± 0.08 0.35	46.0 ± 1.2 5.2	198.9 ± 1.3 6.9			90.4 ± 2.4 10.7	13.6 ± 0.5 2.3

Table 9 *Physical data describing the male physical education students and the different groups of male athletes.*

Sports event	Number of subjects	Age years	Height cm	Weight kg	Maximal values			Pulmonary ventilation l/min	Blood lactate concentration mM
					Oxygen uptake				
					l/min	ml/kg x min	Heart rate beats/min		
Skating cross-country	6	23.4 ± 1.5 2.5	180.4 ± 2.4 4.8	73.2 ± 2.3 4.7	3.41 ± 0.07 0.14	73.9 ± 1.7 3.4	186.2 ± 2.7 8.4	157.9 ± 9.5 18.9	11.0 ± 1.6 2.8
Running long distance	8	26.4 ± 1.2 3.2	176.1 ± 1.9 4.9	64.5 ± 2.0 5.3	4.67 ± 0.15 0.40	72.2 ± 0.8 2.0	191.8 ± 2.9 7.7	145.7 ± 3.3 8.8	14.9 ± 0.7 1.5
Orientierung	16	24.6 ± 0.9 2.9	179.7 ± 1.0 3.8	70.5 ± 2.0 7.9	4.96 ± 0.08 0.31	71.1 ± 1.5 5.8	191.5 ± 2.1 8.1	160.7 ± 3.2 12.5	12.8 ± 0.7 2.2
Bicycling	7	24.1 ± 1.3 3.1	180.4 ± 2.5 8.5	79.2 ± 2.2 5.4	5.55 ± 0.14 0.34	70.5 ± 2.4 3.9	184.9 ± 2.5 6.1	184.5 ± 10.1 4.8	10.6 ± 0.9 1.1
Canoeing	2	22.5	190.5	80.7	5.49	67.7	190.5	165.0	16.4
Rowing	12	24.3 ± 0.6 1.8	189.9 ± 1.0 4.8	86.9 ± 1.4 4.7	6.80 ± 0.07 0.22	68.9 ± 0.9 2.8	189.5 ± 2.1 6.9	179.7 ± 3.7 12.5	10.1 ± 1.0 2.5
Speed skating	7	24.7 ± 1.8 4.4	183.1 ± 2.4 4.9	82.4 ± 2.8 6.7	5.53 ± 0.29 0.72	64.6 ± 2.4 0.8	194.0 ± 5.5 13.5	157.9 ± 9.9 24.3	14.5 ± 1.9 4.6
Handball	13	24.4 ± 0.8 2.8	183.7 ± 1.2 4.3	81.5 ± 2.3 7.8	4.88 ± 0.17 0.6	60.0 ± 1.4 4.8	189.3 ± 3.1 10.8	145.5 ± 4.7 16.4	14.5 ± 0.8 2.5
Bandy	15	24.5 ± 1.2 4.4	178.4 ± 1.7 6.3	75.4 ± 1.7 6.5	4.32 ± 0.11 0.40	56.1 ± 1.5 4.9	189.1 ± 2.1 8.0	127.5 ± 3.8 14.4	11.2 ± 0.7 2.7
Physical education students	20	24.4 ± 0.6 2.3	180.5 ± 1.7 7.3	76.0 ± 2.1 8.9	4.72 ± 0.14 0.62	62.4 ± 1.7 7.5	192.1 ± 1.3 5.5	146.1 ± 4.5 19.6	14.2 ± 0.6 2.8

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EXPERIMENTAL AND CLINICAL STUDIES ON
THE THROMBIN LIKE ENZYME FROM THE
VENOM OF BOTHROPS ATROX. ON THE
PRIMARY STRUCTURE OF FRAGMENT E

1

by

Nils Egberg

Stockholm 1973

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Stockholm Sweden

**EXPERIMENTAL AND CLINICAL STUDIES ON
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The present dissertation comprises a summary of the following separately published papers

- I Egberg N and S Nordström, Effects of Reptilase induced intravascular coagulation in dogs. *Acta physiol. scand.* 1970 79 493—505
- II Egberg N and A Ljungqvist, On fibrin distribution in organs of dogs during defibrination with the thrombin like enzyme from *Bothrops atrox* *Thrombos Res* 1973 3 191—207
- III Egberg N and H Johnsson, Platelet aggregation induced by ADP and thrombin in Reptilase defibrinated dogs. *Thrombos Res* 1972 1 95—112
- IV Egberg N Coagulation studies in patients treated with Defibrase *Acta med scand* In Press.
- V Egberg N On the metabolism of the thrombin-like enzyme from the venom of *Bothrops atrox*. *Thrombos. Res.* In press.
- VI Kowalska Loth, B B Gårdlund N Egberg and B Blombäck, Plasmic degradation products of human fibrinogen II Chemical and immunological relation between fragment E and N-DSK. *Thrombus Res.* 1973 2 423—450

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PREPARATIONS OF CLOT PROMOTING ENZYMES FROM THE VENOM OF BOTHROPS ATROX MENTIONED IN THIS REPORT

All preparations are manufactured by Pentapharm AG Basle Switzerland

1 Reptilase (Haemocoagulase) Available as 1 ml ampoules. The enzymatic activity of this preparation is 1 Klobusitzky unit^{a)} per ml and the clot-promoting activity is derived from a mixture of thrombin like and thromboplastin like enzymes.

2 Reptilase R. Lyophilized preparation containing only the thrombin-like enzyme(s) together with stabilizers to a total amount of 34 mg per ampoule. The clotting activity is equivalent to about 0.1 NIH units of thrombin per mg. The preparation is meant for laboratory use only.

3 Defibrase (initially named Reptilase DEF) Sterile preparation containing only the thrombin-like enzyme(s) together with preservative. Defibrase is available as 2 ml ampoules and is intended for clinical use. Its enzymatic activity is equivalent to about 3.5 NIH units of thrombin per ml. Recent studies have revealed that the clot-promoting enzymes from Bothrops venom for many years have been prepared from two different subspecies Bothrops marajoensis and Bothrops moojeni. The thrombin-like enzymes from these two subspecies are not identical. For studies mentioned in this report batches 403 416 460 477 500 and 550 were used. All batches except 550 contained a mixture of Bothrops marajoensis and Bothrops moojeni enzyme. Batch 550 contained Bothrops moojeni enzyme.

4 Highly purified thrombin-like enzyme. Lyophilized preparations prepared from the venom of Bothrops marajoensis denoted K 126 and DG 144 VIII-XIII. The thrombin-like activity of these preparations were 95 and 120 NIH units of thrombin per mg respectively.

a) One Klobusitzky unit is defined as the quantity of Reptilase which diluted to 1 ml in 0.15 M buffer at neutral pH coagulates 5 ml of oxalated horse blood in 8-10 minutes at 20-22°

INTRODUCTION

It has been demonstrated that several snake venom enzymes possess blood coagulant properties (Mellanby 1909 Eagle 1937 Nahas Denson and Macfarlane 1964 Copley Banerjee and Devi 1973) The clot promoting enzymes of these snake venoms have been found to exert their action on blood coagulation by two different mechanisms (Eagle 1937 Nahas Denson and Macfarlane 1964) Thus one group of enzymes accelerates the conversion of prothrombin to thrombin (thromboplastin like enzymes) while another group causes a direct transformation of fibrinogen to fibrin (thrombin-like enzymes)

Eagle (1937) demonstrated that the venoms of the two South American snakes *Fer de lance* and *Jararaca* or *Bothrops atrox* and *Bothrops jararaca* respectively contained both thrombin and thromboplastin-like enzymes. A method for the isolation of the clot-promoting principle from the venom of *Bothrops jararaca* was described by v Klobusitzky (1935) and by v Klobusitzky and König (1936) A preparation isolated according to this method was later commercially marketed as Reptilase and found to contain both the thromboplastin and the thrombin like enzymes from the *Jararaca* venom (Deutsch 1955 Zurn 1959) By further purification of the Reptilase preparation the thrombin-like enzyme was isolated (Stocker 1970) It was shown that the purified thrombin-like enzyme (initially also named Reptilase) was not inhibited by heparin in the presence of heparin co-factor (Hohnen 1957 Blombäck Blombäck and Nilsson 1957) By means of NH_2 terminal analysis Blombäck (1958) and Blombäck and Laurent (1958) demonstrated that the thrombin-like enzyme clotted fibrinogen by splitting off only fibrinopeptide A in contrast to thrombin which removes both fibrinopeptides A and B It was also shown that the fibrin formed by this thrombin like enzyme initially polymerizes only end-to-end whereas thrombin fibrin polymerizes both end-to-end and side-to-side (Laurent and Blombäck 1958)

The purified form of Reptilase *per se* did not produce fibrinolytic activity when tested on fibrin plates (Blombäck, Blombäck and Nilsson 1957)

IN VIVO EFFECTS OF THE THROMBIN-LIKE ENZYMES FROM BOTHROPS ATROX STUDIED IN DOGS (I AND II)

Previous investigations

In 1936 v Klobusitzky and König suggested that the clot-promoting fraction of the Bothrops jararaca venom could possibly be used for therapeutical purpose in the treatment of haemophilic patients. Several studies on the haemostatic effect of Reptilase (cf. page 7) has since been performed. It has been claimed that in normal subjects injections of Reptilase promptly causes a decrease of both the clotting and bleeding times (Heiss 1954 Bruck 1957 Stacher and Böhnelt 1959 Stenhoff 1960). These effects of Reptilase have been used therapeutically in the treatment of patients with haemorrhagic diathesis (Fleischhacker 1954 Ohlenschläger and Schwalbe 1966) and prophylactically as a haemostatic agent in connection with surgical interventions such as tonsillectomy and prostatectomy (Durante et al. 1962 Sugano and Maruyama 1964) in otherwise normal patients.

The doses of Reptilase given have mostly been 0.25–2 Klobusitzky units once or twice during a 24-hour period (Bruck 1957 Stenhoff 1960 Durante et al. 1962). Depending on the mode of administration, the maximum effect on coagulation and bleeding times is obtained within 3–20 minutes (intravenous administration giving the most rapid effect) and it has been claimed that the effect lasts for at least 24 hours (Stenhoff 1960). Apart from the effect of Reptilase on bleeding and coagulation times no marked influence was found in normal subjects on the platelet count or the concentrations of factor V and VII (Stacher and Böhnelt 1959). These findings indicate that only a low graded intravascular coagulation was induced by the given doses of Reptilase. However Tóth et al. (1967) and Tóth Lévai and Szilágyi (1970) showed that administration of 1–2 Klobusitzky units of Reptilase in connection with the challenge dose of endotoxin markedly increased the intensity of local as well as of generalized Schwartzman's phenomenon.

Reid Chan and Thean (1963) reported on observations in humans bitten by the Malayan pit viper. The victim's blood was found to

remain incoagulable for 2-14 days though no bleeding symptoms were observed. The venom from *Agkistrodon rhodostoma* was later shown to contain a thrombin like activity (Nahas, Denson and Macfarlane 1964, Chan 1964, Chan, Rizza and Henderson 1965). In rabbits the *Agkistrodon rhodostoma* venom rapidly produced a depletion of the circulating fibrinogen apparently without affecting other parts of the coagulation mechanism and without thrombus formation (Regoecci, Gergely and McFarlane 1966). The thrombin-like enzyme was later purified (Esnouf and Tunnah 1967) and commercially named Arvin. Marshall and Esnouf (1968) reported that Arvin injections produced a rapid depletion of fibrinogen in dogs and that dogs defibrinated (in text used equivalent to defibrinogenation) with Arvin were prevented from developing thrombosis when experimental induction was attempted. No toxic side effects were observed after injections of Arvin to dogs. They furthermore showed that injections of large doses of Arvin (4 times the ordinary dose) did produce thrombus formation in the right heart and pulmonary arteries. It has also been shown that impairment of the fibrinolytic system by injection of fibrinolytic inhibitors, prior to the administration of normally harmless doses of Arvin, markedly increases the risk of severe organ damage (Silberman, Potter and Kwaan 1971, Kwaan and Barlow 1971). The fibrinolytic system thus appears to be of major importance for the removal of fibrin deposits after Arvin. Kwaan, Kwaan and Barlow (1971) *in vitro* showed that clots formed by Arvin or Reptilase are more readily digested by plasmin than clots formed by thrombin. Esnouf and Marshall (1968) showed that the fibrin clearing effect of the reticuloendothelial system (RES) did not seem to be as essential in the case of Arvin induced defibrination as in the case of thrombin induced intravascular coagulation (Lee 1962).

Present investigations

The purpose of the first *in vivo* studies on Defibrase was to determine the appropriate dose for uncomplicated defibrination in dogs and to follow the course of defibrination (I). The following study (II) was devoted to a histological examination of the fibrin distribution in organs from dogs defibrinated under conditions similar to those in the previous investigation.

The preparations of purified Bothrops venom were standardized

according to their thrombin-like activity on fibrinogen (Blombäck and Blombäck 1963)

At the time of the first study 1969 (I) the thrombin like enzyme of *Bothrops atrox* was only available as Reptilase R (cf page 7) while in the second study (II) the more convenient preparation Defibrase (cf page 7) was used. These two preparations are identical with regard to thrombin like enzymes (Stocker 1972) In both studies ^{125}I -labelled fibrinogen was used to follow the process of fibrinogen depletion (I) and to perform autoradiographic examinations (II)

Dog fibrinogen used in the studies was prepared by two different techniques (Blombäck and Blombäck 1956 Blombäck Blombäck and Holmberg 1966 Blombäck and Blombäck 1966) Both methods gave a product with a coagulability of about 98 %. The dog fibrinogen was labelled with ^{125}I isotope according to the method described by McFarlane (1963) The coagulability of the fibrinogen after labelling was about 90 %. The specific activity was about $2\mu\text{Ci}/\text{mg}$ in the first study (I) and about $18\mu\text{Ci}/\text{mg}$ in the second (II) and the labelling approximately 1 atom of iodine per mole of fibrinogen. The biological half lives of some of these ^{125}I fibrinogen preparations were graphically calculated and found to be about 2.2 days, which is in agreement with earlier observations (Nordström and Zetterqvist 1968 1969)

Dog fibrinogen prepared as above was also used for immunization of rabbits to produce dog fibrinogen antiserum. The immunoglobulins of this antiserum were precipitated at 1/3 saturation of ammonium sulphate after which the immunoglobulin fraction was subjected to labelling with fluorescein isothiocyanate according to Bergqvist and Schilling (1970)

Experimental conditions

The first study (I) In order to reveal dose response relationship and to perform preliminary determinations of the influence on blood pressure pulse rate and body temperature six dogs were given various doses of Reptilase-R, 7.7–0.9 mg/kg b.w. administered intravenously during one hour Twelve dogs were thereafter given a dose of 1.7 mg/kg b.w. (cf page 7) intravenously during one hour After previous injection of ^{125}I fibrinogen (average $3.8\mu\text{Ci}/\text{kg b.w.}$) the animals divided between four experimental groups

Group No	Administered drugs
I	Reptilase R
II	Reptilase R + Heparin
III	Reptilase-R + ϵ -aminocaproic acid
IV	Reptilase R + Trasylol

One dog out of Group III and one out of Group IV were after the initial infusions given further treatment with Reptilase R by one daily intravenous or intramuscular injection for 10 and 16 days respectively.

Prior to and during the initial infusion of Reptilase R all dogs were anaesthetized by intravenous injections of thiopental sodium. Dogs given long-term treatment with Reptilase R were given the second and subsequent injections rapidly and without anaesthesia.

Blood samples were collected at intervals for determinations of plasma fibrinogen and serum-radioactivity, fibrinogen concentration and platelet count. Factor V and prothrombin concentrations were determined in one and two dogs respectively. Immunodiffusion and immunoelectrophoretic studies were performed in some dogs on serum samples for detection of fibrin(-ogen) degradation products.

The second study (II) In order to provoke if possible a thrombus formation 16 anaesthetized dogs were given a rapid injection of a dose corresponding to the double standard dose of the previous study (0.1 ml of Defibrase/kg b.w. equivalent to 0.35 NIH units of thrombin to 3.5 mg of Reptilase-R per kg b.w.)

¹Nine dogs analyzed by autoradiographic technique were pretreated with ¹²⁵I fibrinogen (average dose 38.4 μ Ci/kg b.w.) 1–2 hours before the experiment. Eight dogs (including three of the dogs analyzed by autoradiography) were examined by immunofluorescent technique. One of the dogs examined by immunofluorescence had been defibrinated by one daily injection of Defibrase (0.1 ml/kg b.w.) for 7 days before the experiment.

The dogs were sacrificed at various intervals 1/2–6 hours after the Defibrase injections by injection of a large dose of thiopental sodium. Immediately after death specimens of lung, liver, spleen, kidney and skeletal muscle were removed for histological examination.

Cryostat sections were prepared for both autoradiographic and immunofluorescent examinations. Due to the relatively low doses of radioactivity used, thick sections (20–60 μ m) were employed and autoradiographs were prepared according to the contact method described by Gross et al. (1951). Sections were prepared for observation of direct immunofluorescence in UV light.

Two control dogs were given physiological saline and thrombin (125 NIH units per kg b w during 30 minutes) respectively and sacrificed 30 minutes after the start of the injections. The dog given physiological saline was examined by both autoradiography and by immunofluorescence. The dog given thrombin was only examined by immunofluorescence in order to test the reactivity of the fluorescein-conjugated antibody in histological sections

In addition to these histological investigations the clearance of endogenous creatinine was determined in four experimental animals (two of these dogs were also used for histological examinations) and in the control dog given physiological saline. Determinations were performed at 30-minute intervals before and after the Defibrase injections.

In all of the dogs given ^{125}I fibrinogen except one the urinary excretion of labelled material was continuously determined.

The process of fibrinogen depletion (I)

The largest dose of Reptilase R (7.7 mg/kg b w) caused a complete fibrinogen depletion within two hours while the smallest dose (0.9 mg/kg b w) caused a 50 % decrease of the fibrinogen concentration within six hours. At the standard dose of 1.7 mg/kg b w a decrease by about 80 % of the initial fibrinogen concentration was obtained within six hours in animals from group I. On the day after the experiment the fibrinogen level was essentially the same as six hours after the Reptilase-R infusion and the pre infusion value was only reached in one dog within three days.

Fibrinogen radioactivity showed a decrease parallel to the fibrinogen concentration. Plasma radioactivity initially fell parallel to the fibrinogen radioactivity but after one hour levelled off simultaneously with an increase of the serum radioactivity. Immunoelectrophoresis of serum sample taken one hour after the Reptilase R infusion revealed presence of fibrin(-ogen) degradation products (FDP). Obviously FDP re-entering the circulation caused the slow decrease of plasma radioactivity and the increase of serum radioactivity.

The platelet count decreased more than 40 % in the dog given the largest dose of Reptilase-R, while at the standard dose the platelet count fell about 25 % (Group I). Factor V activity decreased by about 75 % within seven hours in the dog examined. The prothrombin on the other hand, was unaffected in one dog and decreased only

about 30 % in the other. The decrease of factor V may be due to methodological problems but may also depend on an activation caused by contaminant thromboplastin like enzyme in Reptilase R. The decrease of factor V may also be due to intravascular coagulation, but other indications of such a phenomenon were not observed (clinical signs decrease of platelet count)

In dogs given simultaneous infusions of Reptilase R and heparin (Group II) the course of defibrination and the appearance of FDP was similar to those in dogs from Group I indicating that even *in vivo* heparin does not inhibit the effect of Reptilase R. The platelet count however seemed to be essentially unaffected in this group of animals. The moderate decrease of the platelet count in animals of Group I might be due to aggregation of platelets by traces of thromboplastin like enzyme present in Reptilase R causing an endogeneous generation of thrombin that induces platelet aggregation. In the presence of heparin such an aggregation would be inhibited.

Simultaneous infusions of Reptilase R and fibrinolytic inhibitors, ϵ -aminocaproic acid and Trasylol respectively (Group II and IV) were found to delay but not to inhibit completely the secondary fibrinolytic process. Fibrinolytic degradation products were thus demonstrated immunologically 2—4 hours after the Reptilase R injection. The incomplete inhibition of the degradation of fibrin(-ogen) by the drugs administered might be due to an inadequate inhibition of the fibrinolytic system or to an enzymatic degradation of fibrin(-ogen) not affected by ϵ -aminocaproic acid or Trasylol.

ϵ -aminocaproic acid seemed to potentiate the defibrinating effect of Reptilase-R since two of the three dogs were completely defibrinated within six hours. Platelet count was also less affected in the ϵ -aminocaproic acid and Trasylol group (Groups III and IV) than in the reference group given only Reptilase R (Group I). The latter finding might be due to a thromboplastin inhibiting effect of these compounds previously observed by Nordström and Zetterqvist (1969). No signs of thrombo-embolism were demonstrable in any of the dogs at autopsy. Simultaneous administration of Reptilase R and fibrinolytic inhibitors thus did not seem to severely inhibit the removal of fibrin deposited in the vascular bed during defibrination. This is in conformity with observations of Reid (1965) on patients bitten by the Malayan pit viper. On the other hand several authors have observed thrombo-embolic complications in rabbits given simultaneous injections of EACA and Agkistrodon rhodostoma venom or Arvin (Regoecu

Gergely and McFarlane 1966 Silberman Potter and Kwaan 1971 Kwaan and Barlow 1971) This discrepancy might possibly be due to species differences or to variations in the doses given.

Dogs given long term Reptilase R treatment could be kept at fibrinogen concentrations below 10 mg/ml by one daily injection of Reptilase R. Intramuscular administration appeared to be less effective than intravenous.

The process of fibrin removal (II)

In a few dogs sacrificed 30 minutes and one hour after the Defibrase injections minor fibrin deposits were demonstrated by immunofluorescence in the lung capillaries. On the other hand the glomerulies of the kidneys were not affected in any of the dogs. In agreement with this finding it was further demonstrated that the glomerular filtration rate as measured by clearance of endogenous creatinin did not seem to be significantly affected during the early phase (initial five hours) of Defibrase defibrination.

By the immunofluorescent technique it was also demonstrated that in a few of the dogs sacrificed 30 minutes to five hours after Defibrase injection small amounts of fluorescent material was to be found in the liver sinusoids. Even in the dog sacrificed after eight days of defibrination fibrinogen related material (FR-material) was revealed in the liver sinusoids.

Autoradiographs did not reveal any intravascular fibrin deposits possibly due to a too high background radioactivity. In one dog killed 30 minutes and two dogs killed one hour after the Defibrase injection radioactive material appeared to be accumulated in the red pulp or the spleen at the periphery of the Malpighian corpuscles. These dogs were not examined by immunofluorescent technique.

The fact that only small amounts of FR-material were found either in the spleen or in the liver indicates that during defibrination induced by Defibrase the removal of FR material by RES is of only minor importance. In accordance with these findings, Marshall and Esnouf (1968) demonstrated that the removal of FR material by the RES was of minor importance in dogs after Arvin administration since the incidence of thrombo-embolism did not increase after blockade of the RES.

The secondary fibrinolysis obtained during Defibrase induced defibrination would thus be of major importance for the removal of fibrin. About 80 % of the administered radioactivity was excreted in the urine within eight days. By means of immunoelectrophoresis urine samples were shown to contain FR material (possibly E fragments) giving precipitin reactions with anti-dog fibrinogen serum which also would indicate that the fibrinolytic process is mainly responsible for the removal of fibrin. In accordance with these findings Regöczi, Gergely and McFarlane (1966) Silberman, Potter and Kwaan (1971) and Kwaan and Barlow (1970) found that the incidence of thrombo-embolic complications increased drastically when Arvin was administered to rabbits treated with fibrinolytic inhibitors.

Summary and conclusion

In the first study the elimination of ^{125}I labelled fibrinogen was investigated in dogs after injections of Reptilase R (cf. page 7).

By intravenous administration of Reptilase R, a total depletion of the circulating fibrinogen could be accomplished in dogs within two to six hours without provoking signs of vascular obstruction, bleedings or toxic reactions. A minor decrease of the platelet count was observed with heparin did not affect the defibrination process. Secondary to the defibrination a fibrinolytic process was induced. This fibrinolysis seemed not to be completely inhibited by administration of fibrinolytic inhibitors. No thrombo-embolic complications were observed in dogs given fibrinolytic inhibitors.

In the second study Defibrase (cf. page 7) was injected intravenously to dogs that were sacrificed 1/2–6 hours later. Sections from lung, liver, kidney and spleen were examined by means of autoradiography and immunofluorescent techniques. The observations in the previous study were supported by the histological examinations. Only minor fibrin deposits could be demonstrated in the lung capillaries in a few dogs. The glomerular filtration rate was not markedly affected by Defibrase injections indicating that the defibrination procedure did not produce severe circulatory disturbances. Only small amounts of fibrinogen related material was demonstrated in the liver or the spleen suggesting a minor importance of the reticuloendothelial system for the removal of fibrin after Defibrase injections.

INFLUENCE OF THE THROMBIN-LIKE ENZYME FROM BOTHROPS ATROX ON THE HAEMOSTATIC MECHANISM (III AND IV)

Previous investigations

In vitro studies on platelet aggregation has discovered that presence of small amounts of fibrinogen is necessary for the normal platelet aggregation induced by ADP (Cross 1964 Solum and Stormorken 1965) An inhibitory effect on ADP induced platelet aggregation by FDP has been reported (Kowalski Kopec and Wegrzynowicz 1968 Larnen, Inceman and Marder 1967) However other workers have not found such an inhibitory effect by FDP (Hirsh, Fletcher and Sherry 1965 Cronberg 1968) and it has even been claimed that FDP induces platelet aggregation (Barnhart et al. 1967)

The preparation of Bothrops venom enzymes named Reptilase (cf page 7) was found to induce platelet aggregation and release of ADP (Morse 1967) Reptilase did however not produce clot retraction, which was suggested to be due to absence of release of fibrinopeptide II Reptilase (1-2 Klobusitzky units) administered to patients was found to produce shortened bleeding times (Stacher and Böhnelt 1959) They furthermore stated that Reptilase caused a shortening of whole blood coagulation time but only slightly affected the concentrations of different coagulation factors

Arvin did not affect the concentrations of coagulation factors *in vitro* (Bell, Bolton and Pitney 1968) and in view of earlier findings in animal experiments the therapeutic effect of defibrination induced by Arvin was investigated in patients with thrombo-embolic disorders. From these studies it was reported that Arvin treatment did not affect the platelet count (Bell, Pitney and Goodwin 1968 Sharp et al. 1968) Prentice et al. (1969) however found that the ADP induced platelet aggregation was decreased during the first 24 hours of Arvin treatment but then returned to about pretreatment level. The decrease of the platelet aggregation was concomitant with maximum concentrations of FDP and was thus considered to be due to the inhibitory effect by FDP

Arvin infusions were found to cause a rapid decrease of the fibrinogen concentrations but not to produce any decrease of other coagulation factors (Bell Pitney and Goodwin 1968 Sharp et al. 1968) During Arvin treatment a decrease of the plasminogen concentrations was observed (Bell Pitney and Goodwin 1968 Sharp et al. 1968)

The thrombin like enzyme from *Bothrops atrox* unlike Arvin has in vitro been shown to activate the fibrin stabilizing factor factor XIII (Barlow Holleman and Lorand 1970 Lorand 1972) Recent investigations on the thrombin like enzymes from the two different subspecies *Bothrops marajoensis* and *Bothrops moojeni* have revealed that only the enzyme from the latter subspecies activates factor XIII (McDonagh and McDonagh 1973) The batches of Defibrase (403 416) used for treatment of the three patients in whom factor XIII was determined (see below) contained a mixture of enzymes from these two subspecies (Stocker 1973)

Present investigations

The purpose of the present studies was to disclose the effect of Defibrase induced defibrination on different haemostatic mechanisms. In the first study (III) the platelet function during Defibrase induced Thrombation and in the defibrinated state was investigated in dogs. The effects on different coagulation factors on plasminogen, α_2 macroglobulin and antithrombin III levels were recorded in the second study (IV) by determinations in patients treated with Defibrase for various thrombotic disorders.

Experimental and therapeutic conditions

Seventeen anaesthetized and non anaesthetized dogs (four and thirteen dogs respectively) were injected with 0.10 ml of Defibrase per kg b.w. (III) Platelet rich plasma (PRP) obtained from consecutive blood samples was subjected to analysis of platelet aggregation induced by ADP and thrombin. The effect of reestablishing normal fibrinogen concentration of these samples in vitro was also studied. Bleeding times were determined in three dogs

In order to reveal the effect of FDP on ADP induced platelet aggregation fibrinogen containing plasminogen was digested *in vitro* by addition of urokinase after which the fibrinogen digests were infused to one non-defibrinated and anaesthetized and one defibrinated and non-anaesthetized dog. Platelet aggregation was followed after infusion of the fibrinogen digests.

Thirty-three patients treated with Defibrase for 4–21 for various thrombotic disorders and for anticoagulation in connection with vascular surgery were investigated (IV). Treatment was given in the form of intravenous infusions for one hour 0.01–0.07 ml of Defibrase per kg.b.w. diluted in 100 ml of physiological saline. About half of the patients were only given one infusion per day while the others received two to four infusions per day. The maximum dose was 0.154 ml/kg b.w./day. Patients treated for acute venous thrombosis were given 10 000–12 500 IU of heparin prior to the initial dose of Defibrase in order to achieve immediate anticoagulant effect. Determinations of prothrombin-proconvertin Normotest coagulation factors II V VIII X XII XIII and fibrinogen were performed. Plasminogen α_2 macroglobulin FDP concentrations and spontaneous fibrinolytic activity were determined. The concentration of antithrombin III was analyzed in some patients. Soluble fibrin was demonstrated by the ethanol gelation test and by NH_2 terminal amino acid analysis of fraction I (Abildgaard 1965) of consecutive plasma samples obtained from one patient during the initial 24 hours of Defibrase treatment.

The influence of Defibrase on platelet function (III)

No platelet aggregation was found to be induced by the thrombin like enzyme (K 126) at concentrations corresponding to 0.35–1.00 NIH units of thrombin either in normal or in defibrinated PRP. These findings are in agreement with those of others (Stocker 1972).

After the Defibrase injections a continuous decrease of the fibrinogen concentration was recorded and in all dogs but one there was a total fibrinogen depletion within three hours. An increased concentration of FDP was found already 30 minutes after the Defibrase injection and a maximum amount (1.024–6.400 mg/ml) was reached within 30 minutes to four hours. Soluble fibrin was detected by the ethanol gelation test till the fibrinogen levels decreased below 1 mg/ml.

The first experiments performed on anaesthetized dogs showed that after induction of anaesthesia there was an immediate increase of the platelet aggregation which remained for about two hours after which there was a small decrease of the ADP induced aggregation. These changes occurred irrespectively as to whether Defibrase or physiological saline injections were administered. The changes in platelet aggregation were thus regarded to be mediated by the anaesthesia.

In non-anaesthetized dogs a decrease of the ADP induced platelet aggregation was noted. At fibrinogen levels below 0.5 mg/ml and at FDP concentrations at or above 0.128 mg/ml the ADP induced platelet aggregation was almost abolished. In order to differentiate between the inhibiting effect of low fibrinogen concentrations and high FDP levels fibrinogen was added *in vitro* to PRP samples where the ADP induced aggregation was almost eliminated. By restoring the fibrinogen concentration to about 1.8 mg/ml the platelet aggregation was always normalized to pre-experimental level irrespectively of the FDP concentration. Infusions of FDP to one normal and one defibrinated dog did not influence either ADP or thrombin induced aggregation even when the FDP concentration was at the same level as after Defibrase induced defibrination. Kowalski, Kopec and Węgrzynowicz (1963) pointed out that the inhibition of ADP induced platelet aggregation by FDP could be overcome by higher ADP concentrations. However, after Defibrase defibrination a marked decrease of platelet aggregation was observed even at high ADP concentrations. Thus the decreased platelet aggregation seen during defibrination did not seem to be caused by FDP but rather by the decreased fibrinogen concentrations.

Bleeding times displayed a gradual increase during the initial four hours of Defibrase induced defibrination. This finding suggests that the bleeding time was influenced by FDP which is in accordance with findings of Kowalski et al (1964) and Cronberg (1968).

Thrombin (1-10 NIH units/ml final concentration) used as aggregating agent proved to induce platelet aggregation even at fibrinogen concentrations below 0.5 mg/ml. At these thrombin concentrations the platelet aggregation was reversible and repeated waves of platelet aggregation could be induced by thrombin in the same sample of PRP. One interesting observation was that when defibrinated PRP was preexposed to thrombin aggregation could be induced by ADP alone. Thrombin induced platelet aggregation is believed to be mediated by a release of ADP from the platelets (Haslam 1964). The fact that platelets do not become refractory to small amounts of ADP (O'Brien 1964)

might explain the repeated response to thrombin observed in this study. Thrombin also releases fibrinogen from the platelets and possibly the local concentration of fibrinogen around the platelets becomes high enough to enable them to respond to ADP exposure.

Coagulation studies in patients treated with Defibrase (IV)

The fibrinogen concentration decreased rapidly after infusion of the initial dose of Defibrase (mostly 0.050 ml/kg b.w.) but there was much variation in the degree of defibrination obtained due to the various pretreatment fibrinogen concentrations. The defibrinating effect of the initial dose of Defibrase appeared to last for about 18 hours.

Fibrinogen concentrations below 1.0 mg/ml were regarded as adequate since no excessive bleedings were observed postoperatively at this fibrinogen level and no re-occlusions were noted according to the blood flow measurements and clinical observations. The initial dose of 0.050 ml/kg b.w. which was given to most patients generally proved insufficient as a daily dose to maintain this fibrinogen level. Thus the maintenance dose often had to be increased and when it exceeded 0.070 ml/kg b.w. the Defibrase dosage was divided into two to four infusions per day. There was a direct dose response relationship and there were no signs of resistance to the therapy in any of the patients. After termination of the treatment there was an immediate increase of the fibrinogen values. Within four days most patients reached 50% of their initial fibrinogen values. Five patients observed for five to eight days after treatment did not return to original fibrinogen concentrations within that time range. It was demonstrated that in order to obtain accurate fibrinogen values one had to assure a complete conversion of fibrinogen to fibrin. Methods relying on polymerization rate were often found to give lower values most likely due to interference by FDP.

Fibrinaemia (soluble fibrin) was demonstrated by the ethanol gelation test 1–12 hours after the initial dose of Defibrase and occasionally during the continued treatment. Fibrinaemia was also demonstrated by the presence of NH_2 terminal glycine in the clottable portion of Cohn's fraction I obtained from plasma samples taken one to eight hours after the initial Defibrase infusion. Increase of NH_2 -terminal glycine in the nonclottable portion of fraction I was furthermore demonstrated. This might possibly be due to appearance of clottable high molecular weight FDP e.g. Y fragments.

FDP was demonstrated by the haemagglutination inhibition test (Merskey Kleiner and Johnson 1966) and shown to be present throughout the Defibrase treatment mostly with maximum concentration during the initial 24 hours. However fibrinolytic activity in plasma samples could never be demonstrated on fibrin plates (Ygge 1970). *In vitro* Defibrase has been shown to be capable of partial degradation of isolated fragments of fibrinogen (Hessel and Blombäck 1971 Mattock and Esnouf 1971). Such a degradation of fibrinogen *in vivo* is however less likely due to the low enzyme concentrations.

A decrease of the plasminogen concentration determined by the radial immunodiffusion technique (Mancini Carbonara and Heremans 1965) was found in all the patients investigated. The concentration of plasminogen decreased during the first 24 hours of treatment and thereafter remained at a fairly constant level of about 60 % of the initial plasminogen concentration. There was a good correlation between the amount of fibrinogen removed during the initial 24 hours of treatment and the degree of plasminogen depletion. A correlation was also found between the degree of defibrination and the degree of plasminogen depletion during steady state of treatment.

The concentration of α_2 macroglobulin was determined by the Mancini technique in 14 patients during Defibrase treatment. A decrease though not significant, of the α_2 -macroglobulin concentration was demonstrated. However a correlation between the plasminogen and the α_2 -macroglobulin decrease was found.

From these observations the plasminogen and α_2 -macroglobulin decrease, suggest that the fibrinolytic process obtained secondary to the Defibrase induced defibrination is mediated by an activation of plasminogen. The decrease of α_2 macroglobulin should thus be due to a complex formation with plasmin possibly resulting in a more rapid catabolism.

The concentrations of coagulation factors II V VIII X and XII appeared to be unaffected by the Defibrase treatment. Proconvertin-prothrombin test and Normotest were also unchanged during the treatment. (Dicumarol treatment was instituted two to four days before cessation of Defibrase treatment and affected factors II and X, Normotest and proconvertin-prothrombin tests by the end of the Defibrase treatment.)

On the contrary to other coagulation factors (apart from fibrinogen) the factor XIII concentration, determined by Lorand's fluorescence amino incorporation method (McDonagh, McDonagh and Duckert

1971) decreased significantly in all three patients in whom determinations were made. The decrease appeared within 24–48 hours and thereafter a steady state at 50–60 % of the initial level was obtained in two patients. In the third patient a continuous decrease to 25 % of the initial value was observed. An activation of factor XIII by Defibrase has been demonstrated *in vitro* (Barlow Holleman and Lorand 1970; McDonagh and McDonagh 1973) and these findings indicate that an activation leading to an increased catabolism of factor XIII also occurs *in vivo*.

The concentration of antithrombin III was unaffected by the Defibrase treatment. In one patient who had previously been treated with heparin an increase of the antithrombin III concentration was observed during Defibrase treatment which might possibly be a result of an increased consumption of antithrombin III during heparin treatment (Blombäck et al 1968).

None of the patients treated developed resistance to Defibrase therapy. Such a resistance has however been experimentally induced in dogs (Barlow et al. 1973). In order to investigate if our Defibrase treated patients had developed Defibrase neutralizing antibodies the Defibrase neutralizing capacity of serums from eight patients was tested. None of the serums obtained at the end of Defibrase treatment or obtained five to fourteen months after treatment showed an increased neutralizing capacity. In one patient nausea and vomiting occurred in connection with a Defibrase infusion. The treatment was discontinued as an allergic reaction could not be ruled out. Serum samples from this patient showed, however, no increased Defibrase neutralizing capacity.

Summary and conclusion

It was demonstrated that Defibrase *per se* did not induce platelet aggregation.

Platelet aggregation induced by ADP and thrombin was determined on successive samples obtained during the initial five hours of Defibrase-induced defibrination in dogs. The ADP-induced platelet aggregation proved to be almost abolished at fibrinogen concentrations below 0.50 mg/ml and FDP concentrations above 0.128 mg/ml. As aggregation could be normalized by reconstituting the Γ concentration to 1.80 mg/ml in all samples in which the ADP

FDP was demonstrated by the haemagglutination inhibition test (Merskey Kleiner and Johnson 1966) and shown to be present throughout the Defibrase treatment, mostly with maximum concentration during the initial 24 hours. However fibrinolytic activity in plasma samples could never be demonstrated on fibrin plates (Ygge 1970). *In vitro* Defibrase has been shown to be capable of partial degradation of isolated fragments of fibrinogen (Hessel and Blombäck 1971 Mattock and Esnouf 1971). Such a degradation of fibrinogen *in vivo* is however less likely due to the low enzyme concentrations.

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Observations the plasminogen and α_2 -macroglobulin decrease, suggest that the fibrinolytic process obtained secondary to the Defibrase induced defibrination is mediated by an activation of plasminogen. The decrease of α_2 macroglobulin should thus be due to a complex formation with plasmin possibly resulting in a more rapid catabolism.

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ON THE METABOLISM OF THE THROMBIN-LIKE ENZYME FROM BOTHROPS ATROX

Previous investigations

Purified α_2 macroglobulin was by Lanchantin et al (1966) and Abildgaard (1969) reported to progressively inhibit thrombin. It has also been shown that the inactivation resulted from complex formation between α_2 -macroglobulin and thrombin (Shulman 1954 Lanchantin (1966). Furthermore it was shown that the proteolytic activity of thrombin was inhibited while its esterolytic activity on small synthetic esters was mainly unaffected suggesting that the «active site» was not completely blocked by the association to α_2 macroglobulin (Lanchantin et al. 1966 James Taylor and Fudenberg 1967).

The coagulant activity of the purified thrombin like enzyme from the venom of *Agkistrodon rhodostoma* Arvin, was shown to progressively decrease when incubated with normal human serum (Pitney and Regoezci 1970). The inhibition was shown to result from an association of Arvin to α_2 macroglobulin and possibly also to anti thrombin III.

Present investigations

The main purpose of these studies was to investigate whether Defibrase interacted with serum proteins *in vitro* and *in vivo* and whether physiological inhibitors of proteolytic enzymes were capable of blocking the enzymatic activity of Defibrase. Secondly a preliminary study of the elimination of the thrombin like enzyme and the urinary excretion of the labelled material was performed in dogs.

For these studies a highly purified lyophilized preparation of the thrombin-like enzyme (DG 144) with a thrombin-like activity equivalent to about 100 NIH units of thrombin per mg was labelled with ^{125}I -isotope according to two different methods described by Hunter and Greenwood (1962) and by McFarlane (1963). The former labelled enzyme (in text denoted type A) had a specific activity of about 45

platelet aggregation was almost abolished, it was concluded that the low fibrinogen concentration was of major importance for the decrease of platelet aggregation. This conclusion was also supported by the fact that on infusions of FDP no effect on the platelet aggregation was obtained with the doses of ADP used in this study. On the other hand the bleeding time increased continuously during the defibrination period indicating that it might be affected by the appearance of FDP as well as by the low fibrinogen concentration.

Thrombin on the other hand appeared to induce platelet aggregation regardless of the FDP or fibrinogen concentrations. After pre-exposure of defibrinated PRP to thrombin platelet aggregation could be induced by ADP alone. This finding might be explained by the fact that thrombin releases fibrinogen from the platelet and thereby enables ADP to induce aggregation.

In Defibrase treated patients it was demonstrated that the maintenance dose of Defibrase necessary to keep the fibrinogen concentration below 100 mg/ml was in the range of 0.050–0.150 ml of Defibrase per kg b.w./day. Secondary to the defibrination, an increased fibrinolytic activity seemed to be obtained as judged from the appearance of FDP although no increased fibrinolytic activity could be demonstrated on fibrin plates. A decrease of the plasminogen concentration correlated to a small decrease of the α_2 -macroglobulin concentration was also demonstrated. The latter findings indicate that the fibrin (-ogen) degradation was produced by digestion with plasmin.

Fibrinaemia was demonstrated by the ethanol gelation test and by NH_2 terminal analysis during the initial phase of defibrination.

No signs of generalized disseminated intravascular coagulation were found in terms of consumption of coagulation factors II, V, VIII and X.

Antithrombin III was also unaffected during Defibrase treatment.

Factor XIII decreased to about 50% of the initial value during Defibrase treatment. This is in accordance with earlier *in vitro* findings where Defibrase proved to activate factor XIII.

As judged from the response to treatment with regard to fibrinogen values no patient was resistant to therapy. The fact that the doses of Defibrase had to be increased during treatment might be explained solely by an increased fibrinogen synthesis during Defibrase treatment. An allergic reaction cannot be excluded in one patient. However serum samples from this patient and other patients did not show an increased Defibrase neutralizing capacity *in vitro*.

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Experimental conditions

Due to difficulties in obtaining large quantities of homogeneous fragment E from the serum of patients treated with Desfibrase plasmin digestion of human fibrinogen (fraction I-4) was performed *in vitro* and fragment E was isolated according to the method described by Gårdlund et al (1972)

Fragment E was subsequently subjected to cleavage by cyanogen bromide (CNBr) as described by Blombäck et al (1972) The fragments obtained were separated on Sephadex G 100 in 10% acetic acid The main fragment obtained after cleavage with CNBr was further subjected to tryptic digestion and the tryptic peptides were separated by means of two dimensional low voltage electrophoresis - chromatography (peptide mapping) on thin-layer cellulose plates as described by Blombäck et al (1968) The peptides of the ninhydrin positive spots were eluted for amino acid analysis (Blombäck et al. 1972) The amino acid compositions of the eluted peptides were thereafter compared with those of tryptic peptides of N DSK at corresponding positions on the peptide map

Immunological studies were also performed by means of immunodiffusion (Ouchterlony 1949) and immuno-electrophoresis (Scheidegger 1955)

Chemical and immunological relation between fragment E and N DSK

Immuno-electrophoretic and immunodiffusion studies revealed a partial identity between fragment E and N DSK. It was furthermore shown by immuno-diffusion studies using antisera against isolated chains of N DSK, that parts of the A α and B α chains of N-DSK were present in fragment E

Release of fibrinopeptide A (Blombäck et al. 1966) from fragment E was demonstrated Since N DSK is obtained by cleavage of methionine bonds in fibrinogen by CNBr and fragment E contained 3-4 methionine residues per mole it was decided to treat fragment E with CNBr and isolate the fragments obtained On Sephadex G 100 three peaks of UV absorbing material was obtained The first and largest peak was found to contain material giving a precipitin reaction with anti N DSK serum, showing complete immunological identity with N-DSK. The fragment located in the first peak had a molecular

consisted of three polypeptide chains with molecular weights of 5,500, 6,500 and 8,800 respectively as determined by polyacrylamide gel electrophoresis in SDS (McDonagh et al. 1972). This fragment also proved to have almost the same carbohydrate (Hewitt 1937) and disulphide content (Zahler and Cleland 1968) as fragment E and N-DSK. During thrombin digestion fibrinopeptide A was released from this fragment. Due to the great similarities between N-DSK and this large component of fragment E it was denoted »E-knot».

Immunological identity was demonstrated between »E-knot» and N-DSK and partial identity between »E-knot» and fragment E.

Quantitative NH_2 terminal analysis (Blombäck et al. 1972, Blombäck et al. 1973) of fragment E and »E-knot» were identical. Almost two moles of tyrosine were obtained per mole of »E-knot» suggesting that the NH_2 terminal part of the γ chain was unaffected by plasmin digestion. Alanine was determined to 1.8 moles per mole indicating the presence of fibrinopeptide A. Lysine was the third main NH_2 terminal amino acid, 0.9 moles per mole, probably representing the NH_2 terminal amino acid of the β chain. Appreciable amount of aspartic acid, glycine and valine were also found suggesting a heterogeneity of fragment E.

Peptide mapping of »E-knot» showed that all spots of »E-knot» could be accounted for by spots in N-DSK. Amino acid compositions of the eluted from the peptide map of »E-knot» were found to be in agreement with the known amino acid sequences of tryptic peptides from N-DSK at corresponding positions on the peptide map. The studies suggested the following main structure for »E-knot» (Aa 1 Ala - 51 Met, β 54 Lys - 115 Met, γ 1 Tyr - 58 Lys)₂. The yields of some peptides were however found to be lower than the average yield indicating an incomplete plasmin cleavage resulting in a heterogeneity. Fibrinopeptide A was thus found to be missing in about 40 % of the »E-knot» molecules. The COOH-terminal, 51 Met, of the Aa chain was also missing to the same extent. Low yields of the tryptic peptides β 94 Asn - 115 Met and γ 54 Tyr - 58 Lys suggested heterogeneity even in these parts of the fragment.

The fact that the spot representing the amino acid residue Aa 51 Met of »E-knot» was not present after tryptic digestion of intact fragment E indicates that the Aa chain of fragment E extends outside »E-knot». A difference in the mobilities of the β chains from »E-knot» and fragment E on polyacrylamide gel electrophoresis suggested that the β chain of fragment E extends beyond 115 Met of the »E-knot». Two

additional peaks of UV absorbing material were obtained by filtration of CNBr treated fragment E. The materials of the second and third peaks were found to have molecular weights of about 4 000-5 000 and 500-1 000 and might represent the peptides extending outside »E-knot» in the α and β chains.

Summary and conclusion

After cleavage of fragment E with cyanogen bromide a fragment having a molecular weight of 43 000 and consisting of three polypeptide chains was isolated »E-knot». It was shown that the E-knot contained fibrinopeptide A and the same amount of disulphide bridges and neutral sugars as fragment E and the NH_2 terminal disulphide bond (N-DSK). Furthermore E-knot showed immunological identity with N-DSK. By two dimensional thin layer electrophoresis - chromatography tryptic peptides of »E-knot» were separated. These peptides were subsequently eluted subjected to amino acid analysis and the amino acid compositions compared with the known sequences of tryptic peptides from N-DSK at corresponding positions on the peptide map.

The results suggested the following formula for »E-knot» (α 1Ala 51Met, β 54Lys - 115Met γ 1Tyr - 58Lys) $_2$. Heterogeneity was demonstrated for peptides α 1Ala - 16Arg and 51Met, β 94Arg - 115Met and γ 54Tyr - 58Lys. Sequences extending beyond α 51Met and β 115Met are most likely present in fragment E as these chains of fragment E appeared to be cleaved by CNBr at these positions.

GENERAL SUMMARY

The *in vivo* effects of the thrombin like enzyme from *Bothrops atrox* was investigated in dogs and in humans. Intravenous administration of similar doses of the enzyme produced a rapid depletion of the circulating fibrinogen and a secondary fibrinolytic process in dogs as well as in man. No thromboembolic complications were observed. Histological examinations of dog organs revealed that during the early phase of the defibrination sometimes minor capillary fibrin deposits were produced. However these deposits did not seem to interfere with the circulation. Only small amounts of fibrinogen related material were found in the liver and in the spleen of some dogs indicating a minor importance of the reticuloendothelial system for the removal of fibrin.

No spontaneous bleeding symptoms occurred during the defibrination or in the defibrinated state. A marked decrease of the platelet aggregation was, however demonstrated in dogs during the early phase of defibrination most likely caused by the low fibrinogen concentration not by the increased amounts of fibrinogen degradation products. Bleeding times also increased in dogs during this period and were to be affected by the increased amount of FDP. In humans not defibrinated for several days because of thrombotic diseases coagulation factors II V VIII X and XII were unaffected by the treatment. Factor XIII on the other hand decreased by 40–75 % indicating an activation by the enzyme.

The secondary fibrinolytic process was in dogs found to be delayed but not inhibited by simultaneous administration of fibrinolytic inhibitors. The fibrinolysis caused an increased amount of FDP in the circulation both in dog and man and in humans the maximum concentrations mostly occurred during the initial 24 hours of treatment. The secondary fibrinolytic process caused in humans a decrease of the plasminogen concentration to about 50 % of the initial value.

The thrombin-like enzyme was shown to associate with α_2 macroglobulin *in vitro* and most probably *in vivo*. The complex formed was devoid of clot promoting activity but was able to hydrolyze a synthetic tripeptide substrate. The importance of the α_2 -macroglobulin enzyme complex *in vivo* is obscure. The elimination of ^{125}I -labelled enzyme

was rapid and only about 2 % of the initial radioactivity remained in the circulation after 24 hours.

The primary structure of the plasmic degradation product of human fibrinogen fragment E (m.w 50 000) was investigated. By treatment of fragment E with cyanogenbromide (CNBr) one large fragment (m.w 45,000) showing complete immunological identity with the NH₂ terminal disulphide knot (NDSK) was produced. It was shown that this large fragment, produced by CNBr treatment was a structure included in NDSK with the formula (A α 1Ala - 51Met, β 54Lys - 115Met γ 1Tyr - 58Lys)₂

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VASODILATOR MECHANISMS IN THE SMALL INTESTINE

An experimental study in the cat

BY

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VASODILATOR MECHANISMS IN THE SMALL INTESTINE

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This summary is based on studies reported in the following papers:

- I Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut
B Biber O Lundgren and J Svanvik Acta physiol scand 1971 82 177-190
- II Intestinal vasodilatation in response to transmural electrical field stimulation
B Biber J Fara and O Lundgren Acta physiol scand 1973 87 277-282
- III Intestinal vascular responses to 5 hydroxytryptamine
B Biber J Fara and O Lundgren Acta physiol scand 1973 87 526-534
- IV Vascular reactions in the small intestine during vasodilatation
B Biber J Fara and O Lundgren Acta physiol scand 1973 In press
- V A pharmacological study of intestinal vasodilatory mechanisms in the cat
B Biber J Fara and O Lundgren Accepted for publication in Acta physiol scand
- VI The effects of intestinal vasodilator mechanisms on the rate of ^{85}Kr absorption in the cat
B Biber Accepted for publication in Acta physiol scand

The papers are referred to in the text by their Roman numerals

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INTRODUCTION

Vascular smooth muscle tone and hence also resistance to blood flow is the net result of several different influences. The basal vascular tone is considered to be determined primarily by the myogenic activity within the vascular smooth muscle cells (Folkow 1964) the degree of which differs between various vascular sections. Changes in transmural pressure caused by e.g. a change of arterial blood pressure alters the smooth muscle tone by modifying this myogenic activity. An increased distension for example provides a positive feed back stimulus tending to enhance vasoconstriction.

The remote control mainly exercised by the sympathetic nervous system adjusts the circulation to the requirements of the body as a whole largely regulating total vascular resistance often in competition with local control mechanisms. Sympathetic vasoconstrictor fibres also influence the tone of venous capacitance vessels that provides a dynamic control of the proper filling of the cardiac pump. Furthermore hormonal factors constitute another type of remote control system which in some vascular beds is of importance for the regulation of vascular smooth muscle tone.

At the local level the interplay between the mechanically reinforced myogenic activity and regionally produced chemical vasodilator factors ascertains a blood flow that is closely adjusted to the current demands for oxygen and nutrients in the tissues. Most of our current knowledge about the local chemical control of vascular smooth muscles is based on experiments performed on the skeletal muscle and these studies suggest that certain key substances such as CO_2 , adenosine compounds, potassium and hydrogen ions are of importance (Hilton 1962, Folkow and Neil 1971). An increased tissue concentration of these agents causes a relaxation of vascular smooth muscle. Tissue hyperosmolarity has recently been proposed to be of particular importance in explaining exercise hyperemia (Lundvall 1972).

Not seldom textbook coverage of the local control of circulation implies that the studies of the skeletal muscular circulation may directly apply to all systemic vascular circuits. However several investigations during the last decades suggest that the balance between different mechanisms may vary considerably in different organs. Thus hydrogen ions have been inferred as the principle chemical vasodilator influence in the brain (Fencel et al 1968, Ingvar et al 1968) while adenosine compounds have been suggested to be of particular importance for the control of the coronary vascular bed (Berne 1964). In some glands like the salivary glands the production of specific polypeptide vasodilators, kallidin and bradykinin (Hilton and Lewis 1956, Gautvik 1970) are thought to exert a dominant influence.

As regards the intestinal circulation It has been repeatedly demonstrated that intake of food results in a moderate functional hyperemia. Increasing total splanchnic blood flow 50-300 per cent (Brodie et al 1910 Brodie and Vogt 1910 Herrick et al 1934 Lowenthal et al 1952 Brandt et al 1955 Bensadoun and Reid 1962 Fronek and Stahlgren 1968 Burns and Schenk 1969, Lunderquist Lunderquist and Nommensen 1969). This splanchnic blood flow increase usually takes place at an unchanged or only slightly increased cardiac output: heart rate and blood pressure (Burns and Schenk 1969 Fronek and Stahlgren 1968 Vatner et al 1970). Few studies have however been devoted to the underlying causes of this local increase of gut blood flow. It should be stressed in this context that blood circulation to the gut does not only deliver oxygen and other nutrients to the gastrointestinal tissues but it also constitutes the major transport route for absorbed substances and delivers all the material needed for secretory formation.

It is of considerable interest that absorption, the major function of the gut, is carried out mainly by the epithelial cells of the villi which are situated relatively far away from the mucosal-submucosal arterioles, the main determinants of intestinal flow resistance. Hence it seems less probable that the functional hyperemia of the gut should be induced exclusively by the various agents discussed above which are essentially related to the level of metabolism. Recent experimental work in this and other laboratories has also suggested that more specific mechanisms are at least in part responsible of the vasodilatation seen in the small intestine during digestion and one aim of the present series of experiments was to elucidate this particular question.

Thus in paper I experimental support was presented for the existence of a local nervous vasodilator reflex in the intestinal wall which may be activated by even light mechanical stimulation of the intestinal mucosa. Via this reflex intestinal blood flow may for example be locally increased by the presence of food in the gut lumen. The same local reflex arch could apparently be activated directly by transmural electrical field stimulation (II). A pharmacological analysis of this reflex vasodilatation (I II V) suggested that it was possible to abolish the vascular response by blocking the intestinal receptors to 5-HT (5-hydroxytryptamine, serotonin).

There are recent reports suggesting that other mechanisms than the abovementioned nervous reflex may be of particular importance in explaining the functional hyperemia of the gut. Fara and coworkers (Fara et al 1972) provided experimental support for the view that also the hormones cholecystikinin (CCK) and secretin released as a result of presence of food in the intestine increases intestinal blood flow. In some of the present studies a comparison was made between the

mentioned local neurogenic and these hormonally induced vasodilatations (IV V VI) The pharmacological analysis revealed that also the intestinal vasodilatations caused by the mentioned hormones could be abolished by a 5-HT blockade This suggests that 5-HT may be of common importance for the intestinal functional hyperemia and a particular study was therefore devoted to the effects of 5-HT on the intestinal vasculature as studied both during in vitro and in vivo conditions (III)

The intestinal vascular bed may be considered as a set of parallel coupled circuits supplying the different layers of the intestinal wall Each of these intramural vascular circuits consists in turn of specialized sections coupled in series such as resistance exchange and capacitance vessels (Folkow 1967) The circulatory adjustments caused by the local nervous reflex and the abovementioned hormones were analysed in paper IV with regard to their influence on the series coupled vascular sections utilizing a plethysmographic technique An indirect approach to the study of the intramural parallel-coupled vascular circuits was utilized in paper VI in which mucosal blood flow was evaluated by measuring the rate of absorption from the gut lumen of an inert lipidsoluble radioactive substance ^{85}Kr a technique that has earlier been analysed in more detail with respect to direct blood flow measurements (Svanvik 1973)

Preliminary reports of this series of investigations have been published previously (Biber et al 1970 Biber et al 1972)

METHODOLOGICAL CONSIDERATIONS

The experiments were performed on cats of both sexes deprived of food for at least 24 hours and without any obvious signs of intestinal disease. Anesthesia was induced with ether and maintained with i v chloralose (50 mg/kg b w). The experimental procedures have been described fully in papers I-VI and only a brief review of the methods employed is presented below.

A Operative procedures After tracheotomy a tracheal cannula was inserted and the abdomen was opened through a midline incision. A jejunal segment close to the flexura duodeno jejunalis and weighing 15-30 g was chosen for the experiments. The segment was left in situ with intact vascular supply while the rest of the intestine, the spleen and the greater omentum were removed. Venous outflow from the jejunal segment and its lymph nodes was measured by an optical drop recorder unit, the blood being returned via a funnel connected to the external jugular vein. Arterial blood pressure was monitored from one femoral artery by means of a mercury manometer or a Statham P23 AC pressure transducer. (a) Injections or infusions to the jejunal segment could be made through a cannula placed in a small branch of the superior mesenteric artery. In most experiments the influence of the autonomic intestinal innervation was eliminated by sectioning the nerves surrounding the superior mesenteric artery. The adrenal glands were denervated or entirely excluded from the circulation by ligatures.

Intestinal intraluminal pressure changes could be measured through an intraluminal catheter connected to a Statham low pressure transducer (P23 BC), both ends of the intestinal segments being then tied off.

B Mechanical mucosal stimulation and transmural electrical field stimulation To achieve a mechanical stimulation of the intestinal mucosa a piece of soft plastic tube with blunt ends (length 3 cm; outer diameter 5 mm) was used in most experiments. The plastic tube could be cautiously moved along the jejunal lumen by means of soft strings attached to its ends (I).

Transmural electrical field stimulation (II) was made possible through two specially designed silver electrodes. The inner one was coiled along the full length of a small plastic tube and placed in the lumen to be used as a cathode. The outer one was formed by numerous semicircular pieces of flattened silver wire and so placed that it closely enveloped the intestine. The intestinal segment and the electrodes were placed in a lucite chamber and covered by bodywarm Tyrode solution. Stimulation was delivered through a specially constructed constant current generator operated from a Grass stimulator (model S5).

C. Plethysmographic technique: Plethysmographic studies (III-IV) of the intestinal vasculature were performed using the method described by Folkow *et al* (1963) and Haglund and Lundgren (1972). A jejunal preparation was placed *in situ* into a lucite plethysmograph filled with bodywarm Tyrode solution making it possible to follow continuously changes in tissue volume. Smaller technical modifications described in papers III and IV enabled measurement of changes in tissue volume and capillary filtration coefficient (CFC) also during transmural electrical field stimulation and mechanical mucosal stimulation.

II Absorption of ^{85}Kr : The intestinal uptake of an inert and rapidly diffusible radioactive tracer ^{85}Kr from the intestinal lumen was estimated during intestinal vasodilatation (VI) using a technique described by Biber *et al* (1973 b). Saline containing ^{85}Kr was perfused at a high rate through the jejunal lumen and the tracer amount absorbed was estimated from its appearance in the venous outflow from the jejunum and monitored continuously by means of a well type scintillation counter. As the isotope was almost completely eliminated from the blood after a single pulmonary passage the arterial tracer concentration was negligible so that the ^{85}Kr absorption rate could be calculated from the venous outflow and its tracer concentration.

LOCAL NERVOUS CONTROL OF THE INTESTINAL VASCULATURE

In experiments primarily performed for other purposes. It was noticed that the introduction of a slender plastic tubing into the gut lumen induced a marked increase of intestinal venous outflow (Biber *et al* 1970). This observation represented the starting point of the studies reported in papers I and II. The involvement of the central nervous system in this type of vasodilatation could be excluded first because these experiments were performed on acutely denervated intestinal segments second because no exogenous cholinergic or adrenergic vasodilator fibres have been demonstrated in the cat small intestine (Kewenter 1965). Hence this type of intestinal vasodilatation was likely to be brought about by strictly local mechanisms.

Results and comments

A. Evidence for a local nervous vasodilator mechanism in the small intestine. It was demonstrated in paper I that even light mechanical stimulation of the mucosa of a denervated jejunal segment caused an increase in blood flow amounting to 30-140 per cent of control. Direct visual observation of intestinal segments cut open indicated that the increased blood flow certainly involved the mucosa though also other layers may have been involved. The vasodilatation exhibited a rapid onset and could last for up to 15 min although the mechanical stimulations never lasted more than 30 sec.

A pharmacological analysis reported in paper I strongly suggested that intramural nervous structures were involved in the hyperemia induced by mechanical stimulation of the intestinal mucosa since tetrodotoxin that blocks nerve conductivity abolished completely the vasodilator response to mechanical stimulation. Moreover a similar blockade was accomplished by intraluminal administration of lidocaine another agent blocking nerve conductivity. As regards the transmitter substance in the proposed nervous pathway it was suggested that neither cholinergic nor adrenergic receptors or synapses were involved. It was on the other hand possible to abolish the intestinal hyperemia by bromo-LSD a 5 HT receptor blocking agent. This finding will be discussed in some detail in a following chapter.

If the mentioned intestinal vasodilatation is mediated by an intramural nervous mechanism it should be possible to excite the nerve directly by appropriate electrical stimuli. As reported in paper II an intestinal hyperemia could be elicited by transmural electrical field stimulation with an inner cathode electrode placed in the lumen and an outer one enveloping the intestine which was placed in Tyrode

solution. Stimulation by this procedure elicited a prompt blood flow augmentation with much the same appearance as that induced by mechanical mucosal stimulation and it exhibited identical pharmacological properties. It might be argued that the application of the electrical field could have directly caused a vascular relaxation without necessitating any nervous mediation. If so however the administration of tetrodotoxin or lidocaine would not affect the intestinal vasodilatation induced by electrical field stimulation but these drugs entirely eliminated this response.

Several observations suggest that the mechanical mucosal stimulation and electrical field stimulation evoke vasodilatations via strictly local nervous mechanisms. First acute denervation of the jejunal segment under study did not abolish the vasodilator responses. Second it was shown in paper II that the completely isolated crossperfused intestine still responded with a blood flow augmentation upon electrical field stimulation.

As regards the anatomical arrangement of the nervous structures involved either one of two possibilities a priori seems probable. The arch may be arranged in a manner resembling the peristaltic reflex (Kottgoda 1969) i.e. an intramural reflex arch with one or several synapses. An axon reflex similar to that described for the skin (e.g. Folkow, Ström and Uvnäs 1950) seems to be the other possible arrangement. In order to differentiate between these two possibilities experiments were performed in which cats were operated under sterile conditions all nerves along the superior mesenteric artery being sectioned accomplishing a total postsynaptic adrenergic and presynaptic cholinergic denervation. The animals were allowed to recover and two weeks later when the cut distal parts of the axons had degenerated the animals were again used for investigations as described in paper I. The completeness of the nerve degeneration was ascertained by the total abolishment of intestinal vascular responses to splanchnic nerve stimulation. The vasodilator response to mechanical mucosal stimulation was however unaffected suggesting that structures of the axon reflex type were not involved.

No consistent motility changes as measured from intraluminal pressure or tissue volume alterations were recorded in response to electrical field stimulation. Spontaneous motility was transiently inhibited at the beginning of the stimulation.

To summarize. It is concluded from these experiments that transmural electrical field stimulation evokes a marked intestinal vasodilatation via an intramural nervous reflex arch which during physiological conditions probably is evoked by mechanical mucosal stimulation.

In this connection it should be recapitulated that Ohkawa and

Prosser (1972) recently demonstrated bursts of increased nerve discharge in the submucous and myenteric plexa of the cat small intestine in response to luminal distension. Such an increased discharge was also elicited by means of direct mechanical stimulation of the submucosa and myenteric plexus layers. Ohkawa and Prosser further demonstrated that the increased nerve discharge was accompanied by hyperpolarisation and inhibition of the circular smooth muscles. Such inhibitory neurons to the intestinal smooth muscle layers, also being non-adrenergic and non-cholinergic, can apparently also be stimulated directly through an electrical field (Bülbring and Tomita 1967, Rikimura and Suzuki 1971) the response being blocked by tetrodotoxin and the local anesthetic agent cocaine.

B Effects on consecutive vascular sections The reactions of the intestinal consecutive vascular sections (resistance, exchange and capacitance vessels) during vasodilatation evoked by mechanical mucosal and electrical field stimulation were studied by means of the plethysmographic technique (IV).

Both experimental procedures induced a rapid and large dilatation of the intestinal resistance vessels (see above). Concomitantly there was a dilatation of the capacitance vessels during field stimulation corresponding to a 10-15 per cent increase of the regional blood volume (cf Folkow *et al* 1963). It was not possible to estimate the blood volume changes of mechanical stimulation since the stimulation procedure itself induced large alterations of tissue volume. No attempt was made to differentiate between passive and active factors as regards the recorded dilatation of the capacitance vessels (cf Öberg 1967).

The two different vasodilator procedures caused a considerable increase of the capillary filtration coefficient (CFC) of the intestine. The magnitude of CFC is a function of the number and size of pores per capillary surface area but also of the number of capillaries open for perfusion. This perfused area is determined by the tone of the so called precapillary sphincter sections.

The increased CFC during the abovementioned vasodilatory procedures could thus be brought about by changes in either capillary permeability or in precapillary vascular tone or by a combination of both. No attempts were made to differentiate between these possibilities though no clearcut indications of any increased capillary permeability were seen.

C Effects on intestinal absorption and on parallel coupled vascular sections of the gut A close relationship between the magnitudes of

Intestinal blood flow and intestinal absorption has been demonstrated particularly for lipid soluble substances. In this respect, the mucosal exchange vessels have a key function and circulatory adjustments of this vascular section are usually well reflected in the absorption rate (cf. Svarvlik 1973).

In this investigation the uptake of a radioactive lipid-soluble inert tracer ^{85}Kr was recorded during intestinal vasodilatation induced by electrical field stimulation (VI). The absorption of ^{85}Kr was found to increase in proportion to the blood flow. The observed increase of absorption rate was, however, considerably higher than that recorded during a corresponding increase of total intestinal blood flow induced by isopropylnoradrenaline infusion (Biber et al. 1973b). Furthermore, during the vasodilatation caused by field stimulation a larger fraction of intestinal blood flow became fully equilibrated with the luminal contents compared with control. During isopropylnoradrenaline induced vasodilatation, on the other hand, a decreased equilibrated fraction was observed (Biber et al. 1973b).

These two observations indicate that electrical field stimulation preferentially increases blood flow in the absorptive layers of the intestinal mucosa. This in turn suggests that the studied vasodilatation may be physiologically important during digestion.

HORMONAL CONTROL OF THE INTESTINAL VASCULATURE

It has been suggested since long that intestinal blood flow is influenced by hormones from the intestinal tract (Grossman 1950, Holton and Jones 1960) but this has been demonstrated convincingly first after that the involved hormones became available in a chemically pure form. Thus intravascular administration of secretin and cholecystokinin (CCK) produce intestinal vasodilatation (Fara et al 1969, Ross 1970, Chou et al 1972b, Fasth et al 1973). Furthermore, an increased mesenteric blood flow has been reported in response to i.v. glucagon though the amounts necessary to produce this vasodilatation appear to greatly exceed the physiological dose range for this hormone (Tibblin et al 1970).

The significance of hormonal effects in explaining the post-prandial hyperemia of the gut was investigated by Fara and coworkers (1972). These authors provided experimental support for the view that the functional mesenteric hyperemia is at least in part secondary to the release of CCK and secretin. This was for example suggested by the observation that intraluminal perfusion of solutions containing L-phenylalanine or hydrochloric acid induced an intestinal hyperemia which in all probability was secondary to a physiological release of secretin and CCK (Fara et al 1972).

Since several mechanisms maybe in cooperation can be of importance for the development of the intestinal functional hyperemia it seemed to be of interest to compare the vascular effects caused by the proposed local nervous reflex with that induced by the mentioned hormones. Such a comparison was hence performed as regards the reactions of the consecutive vascular sections in paper IV and as regards the rate of ^{85}Kr absorption in VI. These results are summarized and discussed below while the pharmacological analysis of the vasodilator mechanisms are reported in the next chapter.

Results and comments

A Effects on series-coupled vascular sections The intraarterial or intravenous infusions of physiological amounts of CCK or secretin (4-12 $\mu\text{g/kg} \times \text{min}$) induced a clearcut dilatation of the intestinal resistance vessels (IV). A vasodilatation of a similar magnitude was recorded when a physiological release of CCK and secretin was induced by intraluminal perfusion of L-phenylalanine and hydrochloric acid performed in the same way as by Fara and coworkers (see above). Concomitantly to this dilatation of the resistance vessels a dilatation of the capacitance vessels (mainly veins) occurred being particularly

evident during the intraarterial administration of the hormones which offers a more distinct rise in hormone concentration. It was not further analyzed to what extent this increase of regional blood volume was active as a result of hormonally induced relaxation of the vascular smooth muscles or due to a passive venous distension secondary to the downstream increase of transmural pressure. The effects on the capacitance vessels were quantitatively the same as those observed in connection with the vasodilatations described in the previous chapter.

A considerable increase of the capillary filtration coefficient (CFC) was also demonstrated in response to 1μ infusions of CCK and secretin. For any given blood flow level the increased CFC was always higher than control and sometimes it was apparent before the blood flow increase. At lower infusion rates a CFC increase could be noticed even in the absence of any significant blood flow increase. Corresponding results were obtained during intraluminal perfusion of HCl and L-phenylalanine.

As discussed in paper IV and in the previous chapter the increase in CFC may partly reflect a dilatation of precapillary sphincters partly an increased capillary porosity. No attempts were made to differentiate between these two possibilities. However an increased porosity was suggested by the abovementioned observation that at low 1μ doses of CCK and secretin CFC increased even in the absence of a flow augmentation. This conclusion may also be substantiated by the slow continuous increase in tissue volume seen during the intra-vascular infusion of CCK and secretin. However other mechanisms may explain this observation e.g. an increased mean capillary hydrostatic pressure secondary to precapillary vasodilatation (for a detailed discussion see paper IV).

To summarize CCK and secretin exogenously administered or endogenously released dilate resistance vessels, precapillary sphincters and capacitance vessels in the intestinal vascular bed. The two hormones may also increase capillary permeability by augmenting the size of the capillary pores.

B Effects on intestinal absorption and on parallel-coupled vascular sections. The effect of CCK and secretin on the rate of ^{85}Kr absorption was investigated in the same way as described in the preceding chapter for the local nervous reflex. The results recorded during the hormonally induced hyperemia was similar to that of the nervously produced 1 i.e. an augmented rate of ^{85}Kr -absorption was observed during the vasodilatation. This augmentation was greater than that seen when the same increase in total intestinal blood flow was induced by isopropylnor-adrenaline suggesting that the hormones preferentially increased blood

flow in the absorptive parts of the mucosa

C. Comparative aspects on the different vasodilator responses in the Intestine From the results reported in this and the previous chapter it seems obvious that the vasodilatation induced reflexly or by CCK and secretin involve a pattern of similar reactions in the different series-coupled vascular sections of the Intestine. Moreover, all the vasodilator responses studied induced an increased rate of ^{85}Kr absorption which was greater than that seen during vasodilations evoked by isopropyl noradrenaline. This resemblance in between them is less likely to be incidental, but rather suggests that these particular vascular reactions substantially contributed to the intestinal hemodynamic adjustments occurring in the post prandial situation and that different mechanisms are jointly at hand to fully ascertain the intestinal functional hyperemia. Depending on the nature of the intraluminal contents during digestion, either chemical or mechanical stimuli may dominate. It further seems probable that the release of CCK and secretin which is induced by the presence of food in the Intestine causes an increase of blood flow throughout the Intestine. The local nervous reflex on the other hand induces an intestinal hyperemia that is largely confined to the site of the food bolus.

In a preliminary study a recently developed indicator-dilution technique for measuring intestinal mucosal blood flow and mean transit time (Biber et al 1973a) has been used during vasodilations induced by CCK and secretin or by transmural electrical field stimulation. The method involves intraarterial injections of an intravascular β -radiating tracer and radioactivity measurements by means of a cylindrical detector placed inside the intestinal lumen. The results so far suggest that the mean transit time in the villous part of the mucosa is significantly decreased during the mentioned types of intestinal vasodilations and is even lower than that obtained during isopropyl noradrenaline hyperemia of corresponding magnitude. From previous studies (Svanvik 1973) it has become evident that the absorption rate of ^{85}Kr is well correlated to the mean transit time and hence to the linear blood flow rate. In the mucosa while regional blood volume and diffusion are of relatively less importance. This was suggested to reflect the influence of a countercurrent diffusion exchange which takes place between ascending and descending vessels in the villi and adjacent parts of the mucosa and which delays net intestinal absorption (Lundgren 1967). According to this hypothesis the time available for exchange diffusion is determined by the linear flow rate in the villi and the hindrance to absorption offered by this exchange diffusion would hence decrease at higher linear flow rates. The increased absorption in

paper VI could thus partly be caused by a reduced efficiency of the villous countercurrent exchanger. Further investigations are in progress to elucidate this matter.

PHARMACOLOGICAL PROPERTIES OF THE INTESTINAL VASODILATATION

In the preceding chapters two main types of vasodilatation have been described one apparently induced via a local nervous reflex arch one involving hormonal mechanisms. It is believed that both these mechanisms play a physiological role in the functional hyperemia of the gut. This chapter deals mainly with possible mediator links in the two types of hyperemia. To elucidate this question a pharmacological analysis of the intestinal vasodilatation have been performed particularly with respect to the significance of 5-HT.

Results and comments

A Pharmacological analysis The local vasodilator nervous arch which was activated by slight mechanical stimulation of the mucosa or by transmural electrical field stimulation involved no adrenergic or cholinergic receptor sites either at the effector cells or in the ganglionic synapses (I-III). The same was true for the intestinal blood flow increase induced by CCK or secretin. It was possible however to abolish the vasodilator response to mechanical mucosal stimulation by administration of LSD a 5-HT blocking drug (I). This drug blocked also the vasodilatation induced by transmural electrical field stimulation as was evident from the 4 experiments in which it could be used. Due to the inavailability of 2-bromo LSD 5-HT blockade had to be accomplished by dihydroergotamine (DHE) in the remaining part of this investigation (V). However also DHE completely abolished the intestinal vascular response to local administration of exogenous 5-HT (III) which was utilized as a test of the successfulness of the blockade (Gyermek 1969). DHE have also adrenergic α -receptors blocking properties but first in higher doses than those necessary to block 5-HT receptors.

As reported in paper V the intestinal vasodilator response to transmural field stimulation and to administration of exogenous CCK or secretin was totally abolished by 5-HT blockade utilizing 2-bromo LSD and DHE. However the only 5-HT blocker considered to be fully specific is 5-HT itself. Therefore to obtain further evidence concerning the 5-HT dependence of the vasodilator responses studied a series of experiments were performed with induced tachyphylaxis of the intestinal vascular effects to exogenous 5-HT (V). From these experiments it became evident that a preserved vascular response to 5-HT in the intestine is a prerequisite for the development of the different vasodilatations of this study. Therefore a common physiological role of 5-HT in the development of the hormonally and nervously induced vasodilatations was suggested.

Supporting this hypothesis it was found (paper III) that exogenous

5-HT either given as an intraarterial injection (10 μ g) to the intestine or as a slow i.a. infusion (10-25 μ g/min) produced the same pattern of intestinal vasodilatation concerning the consecutive vascular sections as did CCK, secretin mechanical stimulation of the mucosa and electrical field stimulation (IV). Like these latter vasodilator responses the 5-HT vasodilatation was unaffected by cholinergic and adrenergic pharmacological receptor blockade. However the 5-HT vasodilator response is probably nerve-mediated as it was completely but reversibly inhibited by local nervous blockade with tetrodotoxin (III) or lidocaine (unpublished observations). Actually in some of these experiments an intestinal vasoconstriction was observed in response to 5-HT during nervous blockade.

The results reported above may give the impression that all the different types of vasodilatation are elicited via a common nervous mechanism dependent on an intact 5-HT receptor function. To test this hypothesis experiments were performed in which the effects of tetrodotoxin on the hormonally induced vasodilatations was studied. As reported in paper V tetrodotoxin did not interfere with the hormonal effects. Thus the existence of a vasodilator mechanism common to all types of intestinal hyperemia studied seems less likely. Instead it appears as if two separate mechanisms causing vascular relaxation in the intestine have to be distinguished although the ultimate vascular response exhibits several qualities in common.

B. The possible role of 5-HT for the intestinal hyperemia. Numerous reports concerning 5-HT and its physiological role have been published since the synthesis of the substance was accomplished in 1951 by Hamlin and Fisher (see e.g. reviews by Erspamer 1966, Page 1968, Penttilä 1966, Thompson 1971). In the normal small intestine 5-HT appears to be stored mainly within the intestinal enterochromaffin cells which are scattered among the epithelial cells lining the crypts of Lieberkühn and those covering the villi (cf. Fig. 1). However it also seems probable that 5-HT is normally localized in the myenteric plexus but in quite small amounts usually not detectable by ordinary histochemical methods (Tafari and Ratck 1964, Gershon and Ross 1966, Hammarström et al. 1966, Gershon 1970, Gershon and Altman 1971, Robinson and Gershon 1971).

An unanimous agreement on the functional importance of intestinal 5-HT is yet not at hand. 5-HT has been proposed to participate in the regulation of intestinal motility either as a transmitter released at the smooth muscle cells or in synapses (Kottagoda 1969, Bülbring 1970). The effect of 5-HT on blood vessels are somewhat variable. The in vivo reactions on the vasculature of several organs usually involves

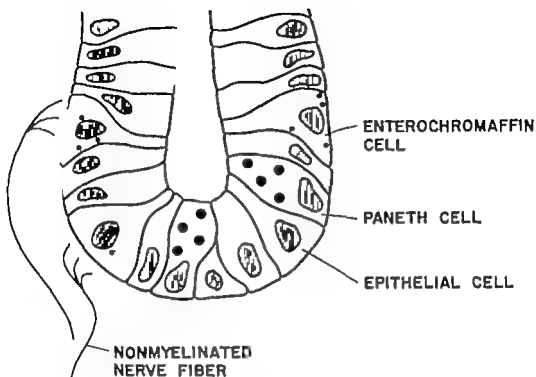


Fig. 1 Epithelium at the base of a crypt in the intestinal mucosa with enterochromaffin cells

a vasoconstriction particularly with respect to larger vessels. This was also found to be the case when cat mesenteric arcuate arteries and veins were studied *in vitro* (III) while 5-HT *in vivo* caused an intestinal vasodilatation in which situation the responses of the small resistance and capacitance vessels dominate.

Concerning the vasodilator mechanisms caused by mechanical mucosal stimulation or by transmural field stimulation, a functional model was proposed in paper V as a basis for further study. In short mechanical stimulation of the mucosa which is likely to occur during digestion excites an intramural neuronal arch (Fig. 2) which involves 5-HT as a synaptic transmitter and possibly also as a stimulus for mucosal nerve endings as a result of 5-HT release from enterochromaffin cells in the mucosa. The electrical transmural field stimulates such

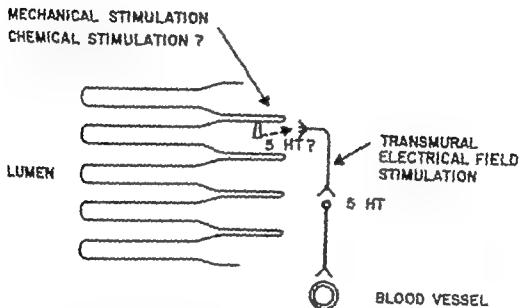


Fig 2 Hypothetical arrangement of a nervous vasodilator reflex arch in the intestine. The number of neurons in each arch is arbitrarily set to two. For details see text.

structures directly evidently before the synaptic 5-HT junction (see Fig 2). Considerable circumstantial evidence for such a model can be presented:

1. Exogenous 5-HT apparently causes a nerve-mediated intestinal vasodilator response which as far as it was possible to investigate on parallel coupled and consecutive vascular sections was similar to that induced by mechanical mucosal or electrical field stimulation (III-IV).
2. The vascular response to mechanical mucosal and electrical field stimulation being also nerve-mediated was reversibly inhibited by pharmacological 5-HT blockade or by vascular 5-HT tachyphylaxis.

3 5-HT occurs naturally in the enterochromaffin cells of the intestinal mucosa where enzymes for its production are available. Fig 1 shows schematically the anatomy of a crypt of Lieberkühn where the enterochromaffin cells with their basal 5-HT containing granulae are positioned in between Paneth cells and ordinary epithelial cells. Small nonmyelinated nerve fibres are located in close contact with the basal membrane of the enterochromaffin cells as was first suggested by Masson (1928) from light microscopy studies and later confirmed by means of electron microscopy techniques (e.g. Gasbarrini et al 1969). Some of the small nerve fibres in the mucosa are obviously of intramural origin arising from the myenteric and submucous plexa (Schofield 1960).

4 Small amounts of 5-HT are also present and actively taken up in the myenteric plexus the precise localization being probably intraneuronal. Additionally Baumgarten et al (1970) have by means of electron microscopy demonstrated non-cholinergic and non-adrenergic neurons called p-fibres (polypeptide fibres) in the myenteric plexus. The axon varicosities of these neurons which establish numerous synaptic contacts are suggested to contain a polypeptide substance (cholecystikinin? secretin?) and 5-HT (Baumgarten et al 1970; Robinson and Gershon 1971).

5 Mechanical mucosal stimulation causes liberation of 5-HT from the intestine into the vasculature (Burks and Long 1966) as does transmural electrical field stimulation in vivo (V). Moreover feeding, probably involving mechanical mucosal stimulation of the intestine leads to an increased 5-HT concentration in the portal blood (Smith 1958; Black et al 1959). Fasting on the other hand minimizing mucosal stimulation, augments the 5-HT content in the intestinal mucosa (Thompson and Campbell 1967) and intestinal wall in general (Gal and Drewes 1961; Lemmer and May 1971).

6 An increased neuronal discharge that inhibits at least intestinal visceral smooth muscle has been demonstrated in response to intestinal distension or other types of mechanical stimulation within the intestinal wall (Ohkawa and Prosser 1972).

The number of neurons in each unit of the proposed nervous arch is unknown as is the nature of the non-cholinergic and non-adrenergic transmitter at the vascular receptor site. It should however in this connection be pointed out that adenosine triphosphate or a related nucleotide has been proposed to be the transmitter substance of the nervously mediated inhibition of gastric smooth muscle (receptive relaxation; Burnstock 1972) and the participation of such a sub-

stance as a transmitter also at the vascular receptor site of the suggested reflex arch in the intestine is not impossible

The possibility that also intraluminal chemical factors can activate the proposed reflex arch is indicated in Fig. 1. Results in agreement with this suggestion was reported by Chou et al. (1972 b) who demonstrated that the intraluminal installation of hypertonic glucose causes a nerve-mediated vasodilatation in the small intestine of the dog. Furthermore, in paper I, it was reported that bile slowly introduced into the gut lumen caused a prolonged intestinal vasodilatation.

As regards the hormonally induced vasodilatation, the present results clearly indicate that the intestinal vascular actions of CCK and secretin require an intact reactivity to 5-HT. However, in contrast to 5-HT itself, the two hormones induce vasodilatation independent of intramural nervous structures. It is difficult to reconcile these observations, but they may be explained by a hypothesis that released CCK and secretin relax vascular smooth muscle in the intestine only when acting together with 5-HT. It has been suggested that enterochromaffin cells and p-fibres in the myenteric plexus contain both 5-HT and poly peptide hormones, and a simultaneous release of these substances may occur (Baumgarten et al. 1970; Robinson and Gershon 1971; Thompson 1971). Also, a reduced content of 5-HT in the intestine has been reported after an intraluminal HCl administration (Resnick and Gray 1962) which has been demonstrated to release also CCK and secretin (Fara et al. 1972). The significance of these latter findings, as well as the true physiological relationship between intestinal hormones and 5-HT has, however, yet to be elucidated.

SUMMARY AND CONCLUSIONS

The present series of experiments was carried out to explore possible mechanisms underlying the post prandial hyperemia of the gut and to investigate the hemodynamic reactions induced by these mechanisms. The results can be summarized as follows:

1. Light mechanical stimulation of the mucosa was demonstrated to cause a localized intestinal vasodilatation obviously through the activation of a local intramural non-cholinergic and non-adrenergic nervous reflex arch (Fig. 2). This nervous structure which could be stimulated directly by applying an electrical field across the intestinal wall was suggested to participate in the development of post prandial hyperemia.
2. 5 HT (5-hydroxytryptamine; serotonin) which is abundantly present in the enterochromaffin cells of the intestinal mucosa and probably to some extent also in the myenteric plexus was shown to be a potent vasodilator of the intestinal vasculature. Judging from a pharmacological analysis 5 HT is directly involved in the reflexly elicited vasodilatation as a neurotransmitter and/or as a stimulus at nerve endings in the mucosa (Fig. 2).
3. The intestinal polypeptide hormones cholecystokinin (CCK) and secretin are known to cause vasodilatation throughout the intestine when released in response to intraluminal chemical stimuli. In the present study it was demonstrated that an intact vascular response to 5-HT is necessary for the vasodilatory effects of CCK and secretin.
4. Pletysmographic investigations showed that the reactions within the consecutive vascular sections (resistance, exchange and capacitance vessels) in the intestine were similar whether a vasodilatation was induced by CCK and secretin or by activation of the intramural nervous vasodilator arch.

Also the different vasodilatory responses in question caused an increased uptake of a lipidsoluble inert radioactive tracer ^{85}Kr from the intestinal lumen. The uptake of the tracer during nervously or hormonally induced vasodilatations was higher than during an intestinal hyperemia of corresponding magnitude induced by isopropylnoradrenaline. It was hence suggested that local chemical and mechanical stimulation of the mucosa leads to such a redistribution of the blood flow in the gut wall as to facilitate absorption.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 402

Glycogen Storage and Depletion in Human Skeletal Muscle Fibres

BY
KARIN PIEHL

STOCKHOLM 1974

Glycogen Storage and Depletion in Human Skeletal Muscle Fibres

AKADEMISK AVHANDLING

som med vederbörligt tillstånd för erövande av filosofie doktorsexamen vid
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AV

KARIN PIEHL

Fil kand.

STOCKHOLM 1974

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 402

From the Department of Physiology Gymnastik- och Idrottshögskolan, Stockholm, Sweden.

Glycogen Storage and Depletion in Human Skeletal Muscle Fibres

BY

KARIN PIEHL

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The present thesis is based on the following papers

- I Gillink P D R B Armstrong C W Seubert IV K Piehl and B Saltin Enzyme activity and fiber composition in skeletal muscle of untrained and trained men J appl Physiol 1972 33 312 319
- II Gillink P D K Piehl C W Seubert IV R B Armstrong and B Saltin Diet exercise and glycogen changes in human muscle fibers J appl Physiol 1972 33 421 425
- III Piehl K Time course for refilling of glycogen stores in human muscle fibres following exercise induced glycogen depletion Acta physiol scand 1974 90 000 000
- IV Piehl K S Adolphson and M Nazari Glycogen storage and glycogen synthesis activity in trained and untrained muscle of man Acta physiol scand 1974 90 000 000
- V Gillink P D K Piehl and B Saltin Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and its repletion rate (For publication 1974)

The papers will be referred to by the following number

INTRODUCTION

The first report describing skeletal muscle composition was published in 1878 by Lorenzini who had noticed the variation in the colour of certain muscles of the rabbit (for reference see Paukull 1904). It was then supposed that the red colour noted in some muscle was due to a rich supply of blood. Kölliker (1850) advanced the hypothesis subsequently confirmed by Köhne (1855) that the pigment was contained within the contractile substance of the fibres (for reference see Busk and Huxley 1853). The substance providing the colour was called myochrome by Mörner (1897) but was later designated myohemoglobin or myoglobin (Günther 1921). Moreover the most active muscles were found to contain more of this pigment (Lester 1871). Using electrical stimulation of red and white muscles Renvier (1873) found that red muscle contracted and relaxed more slowly than white muscles. Grützner (1887) and other investigators studied the behaviour of the muscle during single twitch and during tetanus. Gleib (1887) suggested different metabolism for red and white muscle. In his thesis 1891 Knoll called the fibre protoplasm rich and protoplasmic. Grützner also came to the conclusion that the muscles of higher mammals contained a definite mixture of red and white fibres and that the ratio of the two fibre types governed muscle contractile properties. By staining fibres proving the same contractile properties were grouped in bundles (Denny Browne 1929). These early reports led to the general view that red muscles were associated with a tired repeat work and white muscle with rapid vigorous contraction of brief duration.

In the past three decades intensive histochemical studies have been made on muscle tissue from several species. A number of muscle groups have been examined and at least two or more fibre types have been described. Ogata has suggested three different fibre types based on the following criteria: rate of oxidation, enzyme content and in mammals (Ogata 1956 and b). These authors have recently found differences between the fibre in glycogen content and glycolytic activity by using

exclusively red and white muscle fibre preparations (Ogata 1960). Even though differences in metabolism and substrate content of fibres were described, protein content was found to be the same. This was first determined by Meyer and Weber (1933) and was later confirmed by Bárány and his co-workers (Bárány et al. 1965). This latter group also found greater ATPase activity in white muscles than in red muscles, a finding correlated to a faster rate of contraction by white muscles (Bárány 1967). High and low staining intensity for myosin ATPase determined with the histochemical technique introduced by Padykula and Herman (1955) were also found to correlate to the speed of contraction (Bernard et al. 1971). There have been a limited number of studies of human skeletal muscle. Reports published hitherto have dealt with histochemical findings and few attempts have been made to characterize fibre type or fibre composition in large and varied material. Using histochemical staining for several enzymes, Engel (1962) described the existence of two fibre types: type I and type II. Similar findings were reported by Edström and Nyström (1969) using the same technique. Other investigators have employed different histochemical staining methods to identify various enzymes and substrates. The amount of these has therefore varied as shown in Table I.

Glycogen in muscle was first described in 1858 by Bernard. Thirty years later glycogen was studied by Leloir and his co-workers (1959) and was found to be brought about through an enzymatic system. One of the isolated enzymes, glycogen phosphorylase, was found to be rate limiting and therefore a key enzyme in glycogenolysis. Glycogen as well as the two enzymes involved in its regulation, glycogen phosphorylase and phosphocreatine, have been reported to vary between fibre types in the skeletal muscle of several species including man. A brief summary is given in Table II. Assuming that glycogen phosphorylase and phosphocreatine reflect the ability of the muscle fibre to sustain and utilize glycogen, it seems very likely that fibres with a high level of these enzymes predominantly use glycogen as their source of energy. The white fibres have usually been found to contain both more glycogen and more phosphocreatine (Table II).

Table II Glycogen onto t e d gly og y th t s e d ph ephorylase = tivity i muscle fibres from varying species i ol ding ma light taining or low oti ity, medi m, dark stain ing or high acti ty This subj otive rating i only valid for comparisons within each tudy d a ot be d to make direct ompo is n between v rio s studies

Authors	Species	Methods (hi tochemical and qu ntiti ti)	Fibre types red inter- mediate white
G e r z a d N ik 1958	pige n	m rmi e; que t gly og =	x)
Og ta 1960	r bbit	q a t glyoc	
St in nd Padyk l 1962	rat	PAS	
K gelberg and Ed tröm 1968	rat	PAS, phosphorylase (a b)	
Gillespie Simp e d Edgetton 1970	g i a pig	q nt glycogen	
Peter et al 1972	guin a pig nd rabbit	PAS, ph ephorylase (b) que t glyocgen quant phosphorylase (a b)	()
Lindholm and Pl hl 1974	horse	PAS	
Ha e a d Pearse 1961	rat	ynthetase (I D) phosphorylase (a b)	() ()
St George Stubbs and Blanchaer 1985	guinea pig	ynthetase (I D) phosphorylase (a b) quant synthetase (I D) quant phosphorylase (a b)	
Bocek nd B tty 1986	rat and monkey	ynthetase (I D) phosphorylase (a b) quant synthetase (I D) quant phosphorylase (a b)	
Dubowitz and Pearse 1980	sever l species nd man	phosphorylase (a b)	()
Engel 1962	man	PAS, phosphorylase (a b) ynthetase (I D)	
Ed tröm and Nyström 1968	man	PAS, phosphorylase (a b)	

x) No attempt to distinguish betwe th two red fibre typ e

Conflicting reports have been published on glycogen synthesis. Georg and Naik (1958) reported broad fibres to be glycogen loaded and suggested that these fibres are adapted for rapid and sudden action. Engel (1962) reported type II fibres in human tissue to be rich in both glycogen and the two enzymes. By contrast Sakai and Teuchie (1963) found weak staining intensity for phosphorylase in fibres with large diameters. Stein and Padykula (1962) suggested that variations in glycogen content (PAS staining intensity) reflect various phases in cycle of glycogen storage and release. They consistently greater staining intensity for glycogen in type A (type II) fibres could be interpreted as evidence indicating that these fibres are the most avid synthesizers or that they are the least avid users of glycogen. Stein and Padykula (1962) also emphasized that the PAS staining correlated to quantitative glycogen assay in order to follow glycogen depletion in muscle fibres as a means to determine in which of the fibre types glycogen depletion occurred. A preferential loss of glycogen was found in the white or type A fibres when individual nerve fibres or the entire nerves or muscles of rat were subjected to electrical stimulation (Kugelberg and Edström 1966, Edgerton et al 1970). Exercise in pig and rat was successfully found to produce different glycogen depletion patterns in the fibres (Edgerton et al 1970, Armstrong, Shaphard and Collinick 1973).

On the basis of the foregoing reports summarized in part I Table I and II the fibre types which could be found in human skeletal muscle were outlined. Moreover the glycogen content of the fibres at rest (control) and during exercise (depletion) is not fully known. The important present study was therefore to study and characterize using a large and varied material the fibre types and fibre composition in the skeletal muscle of man. A further aim was to emphasize that under various different controlled conditions in which glycogen is stored in fibres and where the glycogen is gradually depleted in one fibre type rather than other during exercise.

SUBJECTS

Subjects One hundred one healthy men of different ages (17-58 years) with mean weight of 71 (60-85) kg and mean height of 179 (169-183) cm were studied in these investigations. They represented different degrees of training in regard to circulatory function (oxygen uptake) encompassing completely untrained subjects (maximal oxygen uptake of $32 \text{ ml (kg} \times \text{min)}^{-1}$ and extremely well trained subjects (maximal oxygen uptake of $80 \text{ ml (kg} \times \text{min)}^{-1}$). Their mean oxygen uptake was $57 \text{ ml (kg} \times \text{min)}^{-1}$ and their median oxygen uptake $52 \text{ ml (kg} \times \text{min)}^{-1}$.

Muscles examined The quadriceps femoris (vastus lateralis) and the biceps brachii in the determination of fibre distribution and the quadriceps femoris only in the examination of glycogen storage and depletion.

METHODOLOGICAL CONSIDERATIONS

Principle of histohemical evaluation The methodological error of the different methods is given in parentheses in Table III in connection with the method.

Exercise test and determination of oxygen uptake Exercise was performed on a motorically braked bicycle ergometer. Oxygen uptake (\dot{V}_{O_2}) was measured with the Douglas bag procedure over a 10 min period during maximal exercise. The test represented a given percentage of the subject's $\dot{V}_{O_{2\text{max}}}$. Maximal oxygen uptake was determined for each subject using the 1 min ventilation rate (Åstrand & Saltin 1961).

Biochemical technique Muscle samples were taken using the needle biopsy technique (Bergström 1982). Skin and fascia were anaesthetized with 1-1.5 ml of Xylocain and the incision was made with a sharp blade. The anaesthesia was hardly likely to affect biochemical and histohemical assays as samples were always

Table III Brief summary of the methods discussed in the present thesis. Method logic 1 error given in parenthesis 1

A	Assay principle	Method	Assay principle
Quinone titration	Enzymatic activity assay		
Biochemical hydrogen (EC 1.3.1.1)*		Cooperstein, Lezerow and Kufner 1950	Spectrophotometry; reduction of oxidized cytochrome c
Glycogen phosphorylase (EC 2.4.1.11)		Villier-Pleel et al. 1988 modified by Thomas Schliender and Lerner 1988	Incorporation of UDP-glucose ¹⁴ C into glycogen; ± 10 mM G8P
Hokinase (EC 2.7.1.1)		Lowry and Passonneau 1973	Fluorometric reading of NADP coupled reaction
Histochemical staining method			
Alkaline stability in ATP		Podykule and Herman 1955	Localization of phosphatase activity towards ATP indicated by CoCl ₂
NADH dehydrogenase		Novikoff, Shindler and Drucker 1981	Precipitation of diformazan granules
Glycogen synthase		Takuchi and Glenner 1985	Demonstration of polysaccharide synthesis by iodine test; G8P
Glycogen (PAS)		Peters 1981	Demonstration of glycol groups by periodic acid Schiff reaction
Quinone titration	Determinative		
Glycogen (G8P)		Lowry et al. 1984 as modified by Karlsson 1971	Fluorometric readings of NADP coupled reaction
Oxygen uptake (submaximal; max 3%)		Haldane and Priestley 1935	Douglas bag method; Haldane technique

*Enzyme Commission number

obtained from a deep muscle site. The standard sampling site used was 12-18 cm above the patella and 4 cm into the muscle. There are several reasons for using the lateral portion of the thigh for muscle biopsies in addition to the absence of any large vessels or nerves in this region. Glycogen breakdown and blood flow in this muscle as well as muscle temperature have been shown to be related to the bicycle work load performed (Grimby, Häggendal and Saltin 1967, Hermansen, H. Litman and Saltin 1967, Saltin, Gagge and Stolwijk 1968). More recently, Henriksen and Bonde Petersen (1974) found a rectilinear relationship between integrated EMG activity and aerobic power over a wide range of work intensities.

Weighting of samples. Samples for quantitative enzyme determinations were immediately weighed on a Cahn electro balance (this procedure was completed within 3 min) and corrections were made for water evaporation (extrapolation to zero time). Succinate dehydrogenase (SDH) activity was determined in fresh muscle tissue. The samples for determination of glycogen synthetase (GS) and hexokinase (HK) activity were frozen in liquid nitrogen and stored at -80°C . Determinations were made within 2 days. No changes caused by this procedure were found in the activity of these enzymes when comparisons were made with determinations using fresh muscle homogenates.

Samples for quantitative estimations (glycogen) were always immediately frozen in liquid nitrogen while either still in the needle or after having been removed with a forceps. Changes in glycogen content were observed within 10 min at room temperature (Holtman 1967). Samples were stored at -80°C until analyzed.

Mounting of samples for histohemical analysis was performed after orienting the fibre that transverse section could be cut. The mounting medium used was Araldite 502. The freezing procedure was undertaken in liquid nitrogen or isopentane cooled in liquid nitrogen. The latter procedure was used to avoid freeze artefacts in the tissue. Liquid nitrogen is known to produce a gas layer around the sample thereby inhibiting immediate freezing.

throughout the entire muscle sample. Samples were always cut in a cryostat at 20°C.

Subjective rating of PAS staining intensity. A rating system for PAS staining intensity was used in order to demonstrate glycogen distribution in muscle fibres. The rating system comprised four different categories: dark (the fibre most darkly stained for PAS in the sample), moderate, light and negative (no staining). Rating always took place under the light microscope directly from PAS stained slides. Micrographs of samples stained for myo I ATPase were used to identify the fibre types. The reliability of the rating method was evaluated using a photometric method (Edgerton et al. 1974). The correlation ratio of the subjective evaluation of PAS staining intensity to the photometric reading was found to be 0.77. For further discussion see ref. V. The procedure described above is the one suggested by Stein and Padykula (1962) and similar to the one used by Kugelberg and Edström (1966).

RESULTS

Classification of human skeletal muscle fibres (I)

Two fibre types were identified on the basis of staining intensity for myo I ATPase. The two fibre types were designated low twitch (LT) and fast twitch (FT). LT fibres displayed low staining intensity for myo I ATPase and FT fibres were the reverse. Furthermore, ST fibres were more intensively stained for NADH dehydrogenase. The histochemical staining patterns are shown in Fig. 1 (1st page). No third distinct fibre type was found in pieces of the muscle examined. This demonstrates that the fibres displaying both high myo I ATPase and NADH dehydrogenase staining intensity were the same. The staining intensity for NADH dehydrogenase in FT fibres varied strikingly from subject to subject but within the same muscle. The staining intensity was greater in the muscle from the same individual than in the muscle from the same individual. The staining intensity for myo I ATPase was also greater in the muscle from the same individual than in the muscle from the same individual.

Fibre distributions: Wide inter-individual variations in fibre composition were found in both well trained and untrained subjects (I). However variation between the two muscles studied in the same subject seldom exceeded 10-15 %. The coefficient of variation was 4-6 %. This value was based on repeated sampling from the same muscle portion or from the opposite extremity. The proportion of ST fibres was most often found to be greater in muscles from subjects who participated in endurance activities whereas strength trained subjects were the same as sedentary subjects. It is worth noting that this latter group only contained one successful athlete and he had 74 % FT fibres.

Fibre area The area of FT fibres was larger than the area of ST fibres in most subjects. Both fibre types appeared to be larger in the legs than in the arms of most subjects. There was some indication that type of training also affected fibre areas in trained subjects making extensive use of their arms (canoeists and swimmers) or legs (bicyclists). Despite this a linear relationship was still found between the percentage of ST fibres and the relative area taken up by them. Thus the composition of a muscle in terms of the percentage of fibre types is a good indication of the relative areas contributed by each fibre type to the total muscle.

Glycogen storage in muscle fibres (I, II, III and IV)

Evaluated on the basis of PAS staining intensity, no uniform pattern for the distribution of glycogen appeared to exist in the two types of skeletal muscle fibres (I, II, III, IV). However FT fibres were more darkly stained for glycogen than ST fibres when glycogen content of the muscle was less than 80 mmol glucose kg^{-1} (II, III). Staining intensity was at a maximum when glycogen content exceeded this level. It was therefore difficult to determine whether or not there were any basic differences in glycogen content between the fibre types. This was further substantiated by the finding of a PAS staining intensity in some ST fibres which was as high or higher than in FT fibres.

in the cross sectional study (I). It was also obvious that glycogen content was greater in muscles habitually used to the greatest extent.

After previous glycogen depletion by means of exercise the resynthesis process was relatively slow despite a carbohydrate enriched diet and without physical activity as far as possible (III-IV). Almost 48 h were needed to reach pre-exercise glycogen levels. Synthesis occurred at a faster rate in the first 5-10 h after depletion. PAS staining indicated a slight tendency for FT fibres to resynthesize their glycogen more quickly than ST fibres. The percentage of the enzyme glycogen phosphorylase present in the active I form increased when glycogen content was low and a gradual decline occurred with rising glycogen level (IV). Enhanced glycogen accumulation also occurred despite a low level for the I form. The staining intensity for total glycogen phosphorylase (I+D) revealed differences between the two fibre types. However, ST fibres were more darkly stained for the I form after depletion, indicating an enhanced phosphorylation but this could not be related to any greater increase in PAS staining intensity.

Changes in carbohydrate (CHO) content of the diet altered muscle glycogen level (II). Glycogen level was low (mean 43 mmol glucose units \times kg⁻¹) with a fast protein diet and FT fibres were more depleted for glycogen than ST fibres. After mixed and CHO enriched diets glycogen levels were high (mean 84 and 144 mmol glucose units \times kg⁻¹ respectively) and differences in PAS staining intensity would be noticeable between the fibre types.

Influence of training (IV). A comparison of trained and untrained muscle from the same subjects did not indicate a significantly higher glycogen level in trained muscle than in untrained muscle. A higher level of activity for the same glycogen phosphorylase (I+D) and hexokinase was found in trained muscle. No increased rate of glycogen synthesis was found in trained muscle.

Glycogen depletion in muscle fibres (II V)

Glycogen depletion occurred preferentially in one fibre type or the other depending on exercise conditions (V). At varying work intensities (up to work loads requiring $\dot{V}O_2$ max) ST fibres always were the first to lose PAS staining. If work continued some FT fibres were also depleted. The rate of depletion increased with increasing work intensity. The glycogen still remaining in the muscle after exercise at low intensities (non exhaustive) was located in both ST and FT fibres but when exercise had been performed at high intensities (leading to exhaustion) glycogen was found exclusively in the FT fibres. At supramaximal work load (exceeding $\dot{V}O_2$ max) which could not be sustained for continuous periods longer than 1.5 min FT fibres lost glycogen first or at the same time as ST fibres (V). There were always some empty FT fibres. Any glycogen still remaining after such exercise was found in both fibre types. When the pedalling rate was varied thereby varying the rate of contraction no change was seen in this glycogen depletion pattern (V).

A somewhat different glycogen depletion pattern was revealed when almost the same relative work was performed on a bicycle ergometer with varying initial muscle glycogen levels (II). Pre-exercise glycogen levels were low after a fat and protein diet (mean 43 mmol glucose \times kg⁻¹). PAS staining disclosed no darkly stained ST fibres whereas most fibres were lightly stained. FT fibres were more darkly stained overall than ST fibres (17 % dark and the majority moderately stained). During exercise the reduction in muscle glycogen level (PAS staining intensity) occurred preferentially in FT fibres. More FT fibres than ST fibres were negatively stained for glycogen following exercise. After mild and moderate exercise the mean muscle glycogen level amounted to 88 and 144 mmol glucose \times kg⁻¹ respectively. At this time the intensity of PAS staining was dark and similar in both fibre types. Work produced preferential loss of PAS staining in ST fibres under these two dietary conditions. Some FT fibres were also depleted after exercise when starting at the lower glycogen level. This was not the case

when starting on the highest glycogen level. In both cases no fibres were negatively stained for glycogen following exercise and residual glycogen was found in both fibre types.

GENERAL DISCUSSION

Classification of human skeletal muscle fibres

Muscle fibres have been classified in many different ways (see Table I). The use of the terms slow twitch and fast twitch (ST and FT) is based on the contractile characteristics of the fibres. Bárány reported that the specific activity of myosin ATPase is related to the speed of muscle contraction (Bárány 1967). In addition, Guth and Somahe (1968) demonstrated that ectomyosin ATPase activity is correlated to histochemically demonstrable myosin ATPase. The histochemical, biochemical and contractile properties of muscle fibres have been analysed in a pig muscle. A slowly contracting muscle was then shown to correspond to a low level of myosin ATPase activity as demonstrated by both quantitative and histochemical means (Barnard et al. 1971).

Assuming that these results are applicable to human tissue obviously present certain difficulties. However, some reports have been published suggesting that human muscle is composed of two fibre types based on differences in twitch contraction time (Ehret and Goodgold 1966, M. Conner and Thomas 1968). Suchthal and Schmalbruch (1970) correlated differences in contraction rate to high and low myosin ATPase activities. Furthermore, recent results indicate that muscle indicates a correlation between contraction time and quantitatively measured myosin ATPase activity (Edgerton et al. 1971, in preparation) which is in good agreement with data on the human forearm muscle (Bárány 1967). These data provide support for the use of the terms slow twitch and fast twitch muscle fibres even for human muscle tissue.

There is no need to mention the basis for classification is that a continuum of staining intensities from

darkly to lightly stained fibres is revealed in both ST and FT fibres. This overlapping makes it difficult to distinguish between fibre types. Moreover, the oxidative potential of the muscle cell is increased by training thereby changing the training pattern and the basis for classification (Holloszy 1967 Gollnick et al. 1973 a). No such effect on myosin ATPase following physical exercise has yet been reported (Barnard, Edgerton and Peter 1970 Gollnick et al. 1973 a) even though this effect has been noted in animals following cross innervation and electrical stimulation (Closs 1965 Dubowitz 1967 Bárány and Closs 1971 Sträter et al. 1973 Brown et al. 1974). No data on the effects of training on myosin ATPase activity in man are available. It seems reasonable to assume that contractile characteristics are relatively stable as compared to metabolic properties, therefore providing a suitable basis for fibre classification.

One of the problems of the present data is that some types of training are apparently capable of inducing preferential enlargement of either fibre type (Lindberg and Ekblom 1972 Gollnick et al. 1973 a). A linear relationship still existed between ST percentage distribution and relative area. In view of the larger area of FT fibres in muscles from weight lifters and sprinters, a larger number of subjects might possibly alter the shape of the distribution curve. These categories represented only 5 % of the population examined in the present study.

Factors related to glycogen storage

Variations in total glycogen content were found between muscles and subjects both at within different portions of the same muscle (Hultman 1967). Normal values of about 1.2 g x 100 g wet muscle have been reported in the quadriceps femoris (Hultman 1967) but somewhat higher values are usually found in trained subjects (IV Hermans, Hultman and Balke 1967). Resynthesis of glycogen in human skeletal muscle following exercise depletion has been previously analysed (Balke, Hermans and Hultman 1966 Balke, Hermans and Reed-Norlund 1972). According to these studies glycogen levels were greater than the values noted prior to depletion were found.

in muscle after only one day on a carbohydrate enriched diet. These results were not confirmed by the present data (for further discussion see ref. II and III). The highest rate of synthesis observed in this study was $8.4 \text{ mmol glucose units} \times \text{kg}^{-1} \times \text{h}^{-1}$. Compared to a glycogen breakdown of around $30 \text{ mmol glucose units} \times \text{kg}^{-1} \times \text{h}^{-1}$ in exercise at work load demanding about 60-70 % of $\dot{V}O_2 \text{ max}$ which could be performed for 2 h synthesis only amounts to one third of the glycogen consumed in glycolysis.

Glycogen synthetase has the lowest activity of the different enzymes involved in the glycogen synthetic pathway and is therefore considered to be rate limiting. Activity is regulated by the two interconvertible forms of the enzyme: the D form (dependent for activity on the presence of glucose 6-phosphate) and the I form (independent of this activator). The conversion is catalyzed by phosphorylating and dephosphorylating enzymes. Factors affecting the activity of the enzymes regulate the activity of glycogen synthesis. An inverse relationship between the glycogen content of the muscle and synthesis of I form activity has been reported (Duthie 1985). This indicates regulation by feedback mechanism by glycogen itself. In the present study the highest activity was revealed when the glycogen content of the muscle was low. A gradual decline was also noted with increasing glycogen level. However, glycogen content appears to be the only regulating factor in ST fibres despite dark rat innervation intensity for the I form of the enzyme. A higher rate of phosphorylation of glucose into the muscle cell and its phosphorylation is catalyzed by hexokinase, essential for glycogen metabolism. The high hexokinase activity found in muscle may indicate a greater supply of glucose 6-phosphate which is both an activator and a substrate for glycogen synthesis. A number of reports have not validated the present study which has been described previously. It will not be taken into the present discussion (for ref. see Møller and Cribb 1988).

The higher glycogen levels reported previously in trained muscle now bear relation to training and not to diet adjustment. The ability to more than double glycogen content in the muscle

over-shoot or supercompensation phenomenon still awaits explanation. Enhanced glycogen accumulation was noted despite low synthetase I activity. It is obvious that the on and off mechanism for glycogen synthesis is a complex process affected by the other events in the cell.

Factors related to a lentive glycogen depletion during exercise

Glycogen breakdown during different exercise conditions has been widely examined. Results were based on assays of muscle biopsy specimens without muscle fibre differentiation. As indicated by PAS staining, exercise led to selective glycogen depletion in muscle fibres. Thus, true functional changes may be underestimated when evaluations are based on assay of mixed muscle samples.

The extent to which the PAS staining technique provides an exact answer is questionable, especially at maximal staining intensities. PAS staining appears to be at a maximum at glycogen levels exceeding 80 $\mu\text{mol glucose units} \times \text{kg}^{-1}$. Changes in glycogen content above this level cannot be detected as changes in PAS staining intensity thereby making misleading results possible. If FT fibres contain more glycogen than ST fibres, the early appearance of PAS negative ST fibres may be misleading since, for more extensive glycogen breakdown, although masked by the higher initial glycogen content, may have occurred in FT fibres. Glycogen breakdown in ST fibres may also have taken place but impossible to detect by change in PAS staining because of their greater oxidative capacity, thereby enhancing utilization of fat with a glycogen sparing effect.

If the histochemical disappearance of glycogen (PAS staining intensity) from muscle fibres is employed to reflect true changes, it may also be used to discuss the differential recruitment of fibres. Each motor unit is generally regarded as being made up of one type of muscle fibre (Kugelberg and Edström 1968). The relationship between the ability to withstand fatigue and histochemical fibre type has been demonstrated with electrical stimulation (Kugelberg and Edström 1968). Fibres displaying greater staining intensity for oxidative enzymes (red type I or ST fibres)

were found to be less affected by electrical stimulation and were able to maintain contraction for a longer period of time than the white type II or FT fibres. A more rapid loss of glycogen (phosphorylase staining intensity) from type II fibres was also taken as an indication of previous active fibres (Kugelberg and Edström 1968, Edgerton et al 1970a). However, the reverse timing pattern was revealed after physiological exercise (Edgerton et al 1970b, Armstrong, Shepherd and Gollnick 1973). This indicates that ST fibres are recruited first during physiological activation, circumstance possibly ascribable to a lower activation threshold for ST fibres (Henneman and Olson 1965). This kind of stimulation may also possess a lower frequency than the stimulation produced by electrical means so that the activation threshold of FT fibres is not reached. A work containing ST fibres become glycogen depleted and most probably unable to satisfy energy requirements. FT fibres are then gradually recruited (V Gollnick et al 1973b). As already mentioned, tension also appears to be an important factor in fibre recruitment. A shift in glycolysis (PAS staining intensity) from ST to FT fibres was seen when isometric contraction continued for more than 20-25 % of maximal voluntary contraction. The changes were performed (Gill 1974). However, the relationship could be found during bicyclic exercise (V). At this point it may be worthwhile to emphasize that the selective loss of glycogen from ST and FT fibres is not demonstrated by observation from longitudinal fibre recordings (lasting for several minutes or hours). At the end of exercise FT fibres have been shown to be activated for a brief period (Grimby and Hager 1988). This may be the case for brief periods of training in some maximal exercise.

In conclusion, the present study may be said to have shown that only two major fibre types exist in the skeletal muscle of man and that these two fibre types exist in similar amounts of glycogen. Glycolytic and oxidative capacity may be placed somewhat differently in the two fibre types to maintain the different work intensity and the type of exercise required for important metabolic adaptation to the properties of the muscle fibre population to contribute to this phenomenon.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 403

FUNCTIONAL ASPECTS OF
5-HYDROXYTRYPTAMINE TURNOVER
IN THE CENTRAL NERVOUS SYSTEM

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The present thesis is mainly based upon the following eight research papers.

- I Central and peripheral effects of 5-hydroxytryptophan on motor activity in mice. *Psychopharmacologia (Berl.)* 1972, 23, 48-54.
- II Effects of chlorimipramine and protriptyline on the hyperactivity induced by 5-hydroxytryptophan after peripheral decarboxylase inhibition in mice. *J. Neural Transmission* 1973, 34, 101-109.
- III On the role of central nervous system catecholamines and 5-hydroxytryptamine in the p-aminocaproic acid-induced behavioural syndrome. *Br. J. Pharmacol.* 1972, 46, 32-45 (together with T. H. Svensson).
- IV Effects of L-tryptophan on motor activity in mice. *Psychopharmacologia (Berl.)* 1973, 30, 123-134.
- V Effects of p-chlorophenylalanine and monoamine oxidase inhibitor on the 5-hydroxytryptamine in the spinal cord after transection. *J. Neural Transmission* 1972, 33, 211-222. (together with N. E. Andén).
- Effect of chlorimipramine on the rate of tryptophan hydroxylation in the intact and transected spinal cord. *J. Pharm. Pharmacol.* 1973, 25, 926-928.
- VII Effects of isolation and fighting in mice on the rate of synthesis of nor-adrenaline, dopamine and 5-hydroxytryptamine in the brain. *Psychopharmacologia (Berl.)* 1973, 33, 1-17.
- VIII Effects of social stress on the turnover of brain catecholamines and 5-hydroxytryptamine in mice. *Acta pharmacol. et toxicol.* 1974 (in press).

These papers will be referred to by their Roman numeral.

INTRODUCTION

An Indol derivative with pronounced stimulatory effect on smooth muscle was isolated from enterochromaffin cells by Espartero (1946) (for review see Espartero 1954). Shortly thereafter Rapoport, Green and Page (1948) isolated a compound from platelets which, due to its vasoconstrictor properties, was named serotonin. Later the compound isolated by Espartero was shown to be identical with serotonin. Its chemical structure 3-(2-aminoethyl)-5-hydroxyindol (5-hydroxytryptamine, 5-HT) was determined by Rapoport (1949). 5-HT was later shown to be present in brain where it was found to be unequally distributed (Twarog and Page 1953; Axel Crawford and Gaddum 1954) as were the two catecholamines (CA) noradrenaline (NA) (Vogt 1954) and dopamine (DA) (Carlsson, Lindqvist, Magnusson and Waldeck 1958; Bartier and Rosengren 1959; Carlsson 1959; Bartier 1961). By means of the histochemical fluorescence technique introduced by Hillarp and co-workers (Falck, Hillarp, Thilene and Torp 1962; Carlsson, Falck and Hillarp 1962) these three monoamines were shown to be localized intraneuronally, each of them in separate neurons within the central nervous system (CNS). The highest concentrations were found in nerve terminal enlargements, the so-called varicosities.

The suspicion soon arose that these three monoamines may serve as transmitters in the CNS and there is now considerable evidence in support of this view (see e.g. Carlsson 1967; Andén, Carlsson and Haggendal 1969a). The first tentative suggestion that brain 5-HT may have an important role in the control of mental function was based on the early findings that the hallucinogenic drug *d*-lysergic acid diethylamide (LSD-25) antagonized the effect of 5-HT on smooth muscle (Gaddum 1954); and that reserpine, a drug with pronounced antipsychotic and behavioural depressant effects, lowered the concentration of 5-HT in the brain (Brody, Pischner and Shore 1955). These reports were among the most important in stimulating further research on brain monoamines and thereby leading to the development of modern psychopharmacology. However, in spite of extensive studies during the last two decades, the functional role of the 5-HT neurons is still unclear (for review see Chase and Murphy 1973). A major hypothesis, supported by indirect evidence, is that 5-HT neurons may be involved in the control of mood (see e.g. Coppen 1967; Kiehlhol 1968; Roos and Sjöström 1969; Carlsson 1970; Sjöström and Roos 1972).

In this summary of our research correlating behaviour with the 5-HT turn-

over the CNS and investigating some of the mechanisms which regulate this turnover some data relating to the role of DA and NA are also included. A brief introductory review of some of the current concepts concerning the distribution and physiology of the monoamine neurons in brain is first presented.

Distribution of central monoamine neurons

The monoamine neurons in the CNS have been mapped out both by means of the above-mentioned histochemical fluorescent technique and by lesion experiments. The cell bodies of the 5-HT containing neurons are localized mainly in the different raphe nuclei in the brain stem (Dahlström and Fuxe 1965; Heller and Moore 1965; Andén, Dahlström, Fuxe, Larsson, Olson and Ungerstedt 1966). Axons project from these nuclei to virtually all areas of the brain. Including for example the tectal formation, hypothalamus, striatum, limbic forebrain and neocortex. Descending axons project to the grey matter at all levels of the spinal cord, the lumbar and sacral parts being particularly rich in 5-HT terminals (for detail see Fuxe, Håkelf and Ungerstedt 1970; Dahlström, Hägggöden and Årstad 1973).

The majority of the CA neurons also originate from the brain stem. The NA neurons project to widespread areas of the brain and to the spinal cord. The DA neurons project mainly to the neostriatum and to parts of the limbic system (for detailed description see Fuxe et al. 1970; Ungerstedt 1971).

Synthesis of brain 5-HT

The synthesis of 5-HT begins with the hydroxylation of L-tryptophan to 5-hydroxytryptophan (5-HTP) which in turn is decarboxylated to 5-HT (Udenfriend and Titus, W. Isbach and P. P. P. 1956; Udenfriend and W. Isbach 1958). The conversion of L-tryptophan to L-5-HTP catalyzed by the enzyme L-tryptophan-5-monooxygenase (tryptophan-hydroxylase) is considered to be rate-limiting (Jéquier, Lovenberg and Sjoerdsma 1967). The enzyme utilizes oxygen as co-substrate and tetra-hydropteridine as an electron source. The presence of tryptophan hydroxylase in brain (for recent review see Lovenberg 1973) was first demonstrated by Grahame-Smith (1964). This enzyme appears to be specific for L-tryptophan and to be located exclusively in the 5-HT neurons (see e.g. Grahame-Smith 1967; Aghajanian

and Acher 1971; Kuhar, Roth and Aghajanian 1971). It is presumably synthesized in the cell bodies of the raphe nuclei from where it is slowly transported (a few mm per day) to the nerve terminals by an axonal flow (Marek and Neff 1972).

L-5-HTP decarboxylase (for review see Udenfriend, Christenson and Dairman 1973) appears to be identical with an enzyme capable of decarboxylating all naturally occurring aromatic amino acids. Consequently the name aromatic L-amino acid decarboxylase (DC) has been proposed (Lovenberg, Weissbach and Udenfriend 1962). This enzyme occurs in the peripheral and central nervous system as well as in extraneural tissues, e.g. in the kidney, liver and intestine (for review see Hogen and Cohen 1966). It also occurs in the endothelial cell of the brain capillaries where it constitutes a partial barrier between blood and brain for the monoamine precursors 5-HTP and 3,4-dihydroxyphenylalanine (DOPA) (Bertler, Falck, Öman and Rosengren 1966; Bartholli, Constantinidis, Tissot and Pletscher 1971). The regional distribution of DC in the brain (i.e. "beyond" the blood-brain barrier) closely resembles that of the monoamines 5-HT, DA and NA (Bogdanski, Weissbach and Udenfriend 1957; Kuntzman, Shore, Bogdanski and Brodie 1961; McCann, McCann, Hunt and Smith 1965; Lloyd and Hornykiewicz 1972). Recently the DC in the mesencephalon has been demonstrated immunohistochemically to be located in DA- and 5-HT-containing cell bodies but not in their nerve cell bodies (Helfert, Fuxe and Goldstein 1973). Furthermore, lesion experiments reveal that DC in the spinal cord, within the blood-brain barrier, is selectively located in NA- and 5-HT neurons (Andén, Engel and Rubenson 1972). Together these findings indicate that the DC within the blood-brain barrier is located only in the monoamine neurons.

The synthesis of CA (for review see Blaschko 1973) proceeds from L-tyrosine which is hydroxylated to form L-DOPA. This step, catalyzed by L-tyrosine hydroxylase (see e.g. Udenfriend 1966; Weiss 1970), normally appears to be rate-limiting in the synthesis of both DA and NA. The DOPA is decarboxylated to DA. In NA neurons DA is β -hydroxylated to NA. The DOPA-decarboxylase appears to be identical with the 5-HTP decarboxylase (vide supra).

Storage, release and inactivation

The newly-formed transmitter amines synthesized in the monoaminergic neurons are largely taken up and stored in granules, thereby being protected against intra-

neuronal catabolism (vide Infra). The granular storage of these amines is probably essential for the release of transmitter induced by nerve impulses (see e.g. Malmfors 1965; Andén et al. 1969a; Hoggendal and Malmfors 1969; Winer 1970; Dahlström et al. 1973; Smith 1973).

Monoaminergic transmitters released from nerve terminals in the CNS are believed to activate receptors of the postsynaptic effector cells. Apparently these hypothetical receptors have high degree of substrate specificity; thus e.g. 5-HT receptors are not activated by DA and vice versa (Andén 1968).

The action of monoaminergic transmitters at the receptors is terminated by catabolism outside the monoaminergic nerve terminals and probably of more importance by active re-uptake of the transmitters into the presynaptic nerve terminal followed by catabolism or re-uptake into granules (for review see Andén et al. 1969a; Carlsson 1970; Iversen 1970). The active re-uptake mechanisms i.e. the membrane-pumps mentioned above show certain degree of substrate specificity towards their normal transmitter; the different monoamine neurons; thus 5-HT at low concentrations ($\sim 0.1 \mu\text{M}$) is preferentially taken up by the membrane pump of 5-HT neurons. This relative specificity is however lost at higher concentrations of transmitters; e.g. high concentrations of 5-HT are taken up by CA neurons (for review see Snyder, Shaskan and Kuhar 1973).

5-HT is chiefly catabolized both inside and outside the 5-HT neurons to monoamine oxidase (MAO) to 5-hydroxyindole-3-acetaldehyde which is then converted to 5-hydroxyindole-3-acetic acid (5-HIAA) by an aldehyde dehydrogenase (Tilus and Udenfriend 1954; Sjoerdsma, Smith, Stevenson and Udenfriend 1955; for review see Blaschke and Levine 1966). MAO is widespread in peripheral tissues and in the CNS where it is intracellularly bound to mitochondria. Both glial cell and nerve cell contain MAO (for review see Tipton 1973). The intraneuronal catabolism of CA also occurs mainly by MAO. However CA are also catabolized in the extracellular space by catechol O-methyltransferase (COMT) (for review see Andén et al. 1969a; Jonason 1969; Shorman 1973).

Regulatory mechanisms

The metabolism, storage and release of 5-HT in the CNS seems to be regulated by several different mechanisms. The nature of these mechanisms is only partially elucidated.

The tryptophan hydroxylase is not normally saturated with tryptophan (Eccleston, Ashcroft and Crawford 1965; Fernstrom and Wurtman 1971; Grahame-Smith 1971; Carlsson and Lindqvist 1972). Consequently the rate of 5-HT synthesis depends both on the concentration of tryptophan in 5-HT neurons and on the activity of tryptophan hydroxylase.

L. Tryptophan is taken up into brain and synaptosomes by means of carrier mechanisms. Several other neutral amino acids compete for these uptake mechanisms (Guroff and Udenfriend 1962; Blasberg and Lajtha 1963; Grahame-Smith and Parfitt 1970; Kleiy and Soukes 1972). Consequently the uptake of tryptophan into the brain is dependent on the relation between the plasma concentrations of both free tryptophan (i.e. not bound to albumin) and of other neutral amino acids (Fernstrom and Wurtman 1972). A study of the uptake of tryptophan into only 5-HT neurons has not been possible; thus it is not known whether this uptake differs from that occurring in other cells within the CNS.

The tryptophan hydroxylase activity seems to be partially dependent on the flow of nerve impulses in the 5-HT neurons. Support for this view is provided by the findings that the activity of tryptophan hydroxylase was moderately decreased in acute lesion experiments (Carlsson, Bédard, Lindqvist and Magnusson 1972a; Carlsson, Lindqvist, Magnusson and Atrak 1973b; Schubert 1973) and increased during the electrical stimulation of raphe nuclei (Shields and Eccleston 1972). Since these changes in enzyme activity are rapid, they probably reflect different degrees of activation of the enzyme rather than variations in the synthesis of new enzyme.

Tryptophan hydroxylase activity has also been proposed to be regulated by end-product inhibition, i.e. by variations in the intraneuronal concentration of 5-HT (Macon, Sokoloff and Glowinski 1971; Hanson, Bourgois and Glowinski 1973; Schubert 1973). Results from *in vitro* experiments are however contradictory in this respect (cf. Grahame-Smith 1964; McGeer and Piers 1969; Jéquier, Robinson, Lovenberg and Sjoerdama 1969; Hanson *et al.* 1972).

At least under certain conditions part of the 5-HT seems to be catabolized intraneuronally without having been functionally active. This view is supported by results from behavioural experiments (Grahame-Smith 1971) and lesion experiments (Bédard, Carlsson and Lindqvist 1972). Thus at least two different compartments for newly-formed 5-HT may exist: one functionally active and the other functionally

inactiv (cf Grahame-Smith 1973) Such a compartmentalisation may well have the regulatory function of compensating for variations in the rate of 5-HT synthesis which occur independently of the functional need for transmitter

Drugs which by different modes of action are supposed to activate central 5-HT receptors depress the firing of raphe cells. This inhibitory effect may well be mediated by a feed-back mechanism which is activated by an increased stimulation of pre- or postsynaptic 5-HT receptors (for review see Aghajanian and Haigler 1973). Such a feed-back mechanism may serve the purpose of counteracting the change in activity of the 5-HT receptors.

Objective of the present study

Drugs with antidepressant properties and well documented effects on the synthesis or inactivation of 5-HT were examined. Attempts were made to evaluate their effects at the 5-HT receptors by means of the behavioural changes which they induce in animal. Furthermore the possible relationship between the impulse activity of 5-HT neurones and the rate of synthesis of 5-HT was examined. The study has implications for clinical research on the possible involvement of central 5-HT neurones in mental diseases. Major approaches used in such clinical research are: 1) to evaluate the therapeutic effects of drugs which by different modes of action interfere with the neurotransmission in 5-HT neurones; knowledge of the effects of these drugs on the 5-HT receptors is then of fundamental importance. 2) to search for correlations between mental symptoms and changes in the rate of turnover of 5-HT in the CNS. The significance of such changes in turnover depends on the extent to which they reflect the functional activity of the 5-HT neurones.

METHODOLOGY

The methods used in the experiments are described in detail in the appropriate papers. A brief survey of these methods is given below.

Subjects

Male mice (NMRI strain) weighing 20-25 g and male Sprague-Dawley rats weighing 180-220 g were used.

Substances used

L-Tryptophan (Ajinomoto Co. Inc., Tokyo)

DL-5-Hydroxytryptophan (Ajinomoto Co. Inc., Tokyo)

DL- α -Methyl-metatyrosine (α -MMT, Regis Chemical Co., Chicago)

Chlorisipramine HCl (Ciba-Geigy, Basel)

Protriptyline HCl (Merck Sharp & Dohme International, Haarlem)

Nialamide HCl (The Swedish PET or Ltd., Näsbypark)

Reserpine (The Swedish Ciba Ltd., Stockholm)

DL-p-Chlorophenylalanine methyl ester HCl (PCPA); (H69/17, Hässel, Göteborg)

DL- α -Propyldopac tartrate (H22/54, Hässel, Göteborg)

DL- α -Methyl-p-tyrosine methyl ester HCl (α -MT); (H44/68, Hässel, Göteborg)

L- α -Hydroxy- α -methyl- β -(3,4-dihydroxyphenyl) propanoic acid (WAK 486, Merck Sharp & Dohme International, Haarlem)

3-Hydroxybenzyl hydrazine HCl (NSD 1015, Smith & Nephew Research Ltd, Harlow)

5-Hydroxypyrazolo-(3,4-d)-pyridine (Allopurinol, Burroughs Wellcome & Co., London)

All drugs were injected in approximately neutral solutions by the intraperitoneal route.

Motor activity

The motor activity was in our earlier experiments measured by means of Animax activity meters (Farad Inc., Sweden). These are based on a tuned oscillator system which becomes off-tuned when a mass is introduced into its electro-

magnet field (for detailed description see Svensson and Thlense 1969). In later experiments the motor activity was measured by means of M/P 40 Fc Electronic Motility Meters (Motron Products Sweden). These are based on 40 photoconductive sensors arranged in 5 rows of 8 cells with center to center distance of 40 mm. The cells are covered by translucent plastic box. The light source is a regular incandescent lamp mounted 115 cm over the photoconductive sensors. In all experiments the motor activity of one group of three mice was measured at a time. The recordings were started 30 sec after inserting the animal in the test cages. The motor activity was in one experiment recorded continuously for 6 h and registered as counts from the activity meters per 10 min (paper III). In other experiments the motor activity was recorded during 20 min and registered as counts per 20 min. The animals were housed under normal lighting conditions. The measurements of motor activity were generally performed between 10:00 a.m. and 3:00 p.m.

Spinal transections

The descending 5-HT containing neurons have their cell bodies in the brain stem. A transection of the spinal cord will therefore block the flow of nerve impulses and the supply of tryptophan hydroxylase to the nerve terminals below the transection (see Introduction). Rats were spinalized at the midthoracic level under ether.

A care was taken to maintain their normal body temperature by changing the environmental conditions. The urinary bladders of these spinal rats were emptied twice daily by gentle pressure on the abdomen. Unoperated rats serving as control were given ether anaesthesia unless otherwise stated.

Biochemical determinations of monoamines, their precursors and catabolites

Tissues were homogenized in 10 ml 0.4 N perchloric acid containing 20 mg disodium ethylene diamine tetraacetate and either 2 mg ascorbic acid or 5 mg $N_2S_2O_5$. The compounds were isolated from the extracts on columns of cation exchange resin and assayed spectrophotofluorimetrically.

In earlier experiments NA and DA were isolated on strongly acidic cation exchange column (Dowex 50W X-4) (Berler, Carlsson and Rosengren 1958 as modified by Carlsson and Lindqvist 1962) and 5-HT was isolated on weakly

acidic cation exchange column (Amberlite CG50 type 1) (Bertler 1961 as modified by Andén and Magnusson 1967); and assays were made for NA (Bertler et al 1958) DA (Carlsson and Waldeck 1958 as modified by Carlsson and Lindqvist 1962) and 5-HT (Andén and Magnusson 1967).

In later experiments all compounds were isolated on a single column of the Dowex 50W x8 (Atack and Magnusson 1970; Lindqvist 1971; Kehr, Carlsson and Lindqvist 1972) and assays were made for tryptophan (Bédard et al 1972) 5-HTP, 5-HT and 5-HIAA generally using native fluorescence (see Atack and Lindqvist 1973) tyrosine (Waalikes and Udenfriend 1957) DOPA (Kehr et al 1972) DA (Atack 1973) and NA (Bertler et al 1958).

Model used for the estimation of rate of synthesis of transmitter and of impulse activity in central monoamine neurons

Recently a method has been developed for determining simultaneously the rate of hydroxylation of tryptophan and of tyrosine *in vivo* (Carlsson, Dahl, Kehr, Lindqvist and Atack 1972b). Under normal conditions these hydroxylations are considered to be rate-limiting in the synthesis of 5-HT and CA respectively (see Introduction). The products of these hydroxylations, 5-HTP and DOPA respectively, are normally rapidly decarboxylated and are not readily detectable in the brain. After blockade of DC in the CNS, the concentrations of 5-HTP and DOPA increase linearly. These accumulations of 5-HTP and DOPA appear to provide reliable measures of the rates of synthesis of 5-HT and CA. The concentrations of 5-HTP and DOPA in CNS were determined 30 min after the administration of NSD 1015 (100 mg/kg). This drug inhibits both DC and MAO and is largely without effect on gross behaviour in animal (see Carlsson et al 1972b). Variations in the rates of synthesis of 5-HT and CA were estimated using either the above method (papers VI and VII) or in earlier experiments (papers III and V) by determining estimated accumulations of 5-HT, DA and NA after inhibition of MAO (Neff and Tizer 1968; Neff and Cost 1968).

Impulse activity in the different monoaminergic neurons in the CNS is considered to be reflected by the rates of depletion of 5-HT, DA and NA after complete inhibition of their biosynthesis. This will be supported by the findings that electrical stimulation of NA- or 5-HT containing cell bodies accelerates the depletion of NA and 5-HT respectively, whereas the depletion of 5-HT, DA

and NA distal lesion of the respective neurons is almost completely antagonized (Andén, Fuxe and Hökfelt 1966; Andén, Corrodi and Fuxe 1969b; Andén, Corrodi, Fuxe and Ungestedt 1971; Korf, Aghajanian and Roth 1972; see also paper V). Furthermore, there is good agreement between the effects of drugs on the rate of depletion of 5-HT after synthesis inhibition and their effects on the impulse activity of 5-HT neurons as determined from direct recordings in the raphe (compare e.g. Andén, Corrodi, Fuxe and Hökfelt 1968; Corrodi and Fuxe 1968 with Aghajanian, Foote and Sheard 1970; Sheard, Zolovick and Aghajanian 1972). In the present study the rate of depletion of 5-HT in brain was determined after the administration of α -propylidopocetamide (H22/54) 500 mg/kg, an inhibitor of both tryptophan- and tyrosine hydroxylase (Carlsson, Corrodi and Waldeck 1963), and the rates of depletion of DA and NA were measured after the administration of DL- α -methyl-p-tyrosine methylester HCl (α -MT) 250 mg/kg, which is a fairly specific inhibitor of tyrosine hydroxylase (Spector, Sjoerdsma and Udenfriend 1965; Svensson and Waldeck 1970).

STUDIES ON THE ROLE OF 5-HT IN THE BEHAVIOURAL EFFECTS OF ANTIDEPRESSANT DRUGS

In the treatment of mental depression drugs inhibiting MAO as well as tricyclic drugs of the imipramine type are well established to be effective. L-Tryptophan and 5-HTP the precursors of 5-HT are reported by some investigators to have antidepressant properties whereas others have found them to be ineffective in this respect. The elevation of mood induced by these various groups of drugs has been proposed to result largely from their common ability to facilitate neurotransmission in central 5-HT neurons: MAO inhibitors by blocking the catabolism of 5-HT the tricyclic drugs by blocking the membrane pump in 5-HT neurons and the precursors by increasing the formation of 5-HT (for review see e.g. Coppen 1973; Murphy, Baker, Kolfin and Bunney Jr. 1973; Brody, Sack and Siever 1973). The effects of these various groups of drugs on the 5-HT receptors cannot however be predicted with any certainty. Few of the complex regulatory mechanisms which seem to govern neurotransmission in 5-HT neurons (see Introduction).

There are no sensitive models available for estimating variations in activity of 5-HT receptors. In consequence the effectiveness of the various groups of antidepressant drugs on the 5-HT receptors is poorly documented. Evidence that the groups of drugs mentioned above do act on 5-HT receptors is limited to treatments which result in markedly-increased concentrations of 5-HT in the CNS: complete inhibition of MAO and the combined treatment of MAO inhibitors with precursors of 5-HT (see e.g. Corrodi 1966; Meek, Fuxe and Andén 1970). When using such treatments the additional administration of tricyclic antidepressant drugs results in further increase in the activation of the 5-HT receptors (Carlsson, Jonason, Lindqvist and Fuxe 1969c; Meek et al. 1970).

The various groups of antidepressant drugs differ in their effects on the behaviour of laboratory animals (vide infra). Attempts were made in the present investigation to determine to what extent these differences in effect on behaviour reflect variations in activity of central 5-HT receptors. This by varying the effectiveness of the various drugs on the 5-HT receptors. All were used in all experiments and the influence of the various drug treatments on their motor activity was recorded.

Exogenous 5-HTP is rapidly decarboxylated to 5-HT both in peripheral and in central tissues which contain DC (Udenfriend, W. Isbach and Bogdanek, 1957). The pharmacological effects of 5-HTP are generally considered to be mediated via the 5-HT so formed (see e.g. Corne, Pickering and Warner, 1963). The development of DC inhibitors e.g. MK 486 which poorly penetrate the blood-brain barrier and thereby inhibit peripheral but not central DC (Porter, Watson, Titus, Tatars and Byer, 1962; Bartholini and Witscher, 1969) has made it possible to block the formation of 5-HT from 5-HTP selectively in peripheral tissues. When given alone 5-HTP is reported to induce sedation or behavioural depression in mice and rats (for review see Montegazzani, 1966). Only a few quantitative studies are reported of motor activity in mice treated with 5-HTP. Using photocell counter technique Brown (1960) reported reduced motility in mice treated with DL 5-HTP in doses from 0.1 to 100 mg/kg, whilst Smith and Dews (1962) found no changes in mice treated with DL 5-HTP in doses between 10 and 1000 mg/kg.

DL 5-HTP in doses between 6.25 and 50 mg/kg had no significant effect on motor activity either when given alone or in combination with MK-486. Higher doses of DL 5-HTP (100-800 mg/kg) caused a reduction in motor activity when alone but dose-dependent increase when given to animal pretreated with MK 486. The increased motor activity was accompanied by rapid head movements in animal given the highest doses of 5-HTP (400-800 mg/kg). Some of these animals also displayed tremor in their forelegs and abduction and hyperextension of their hind legs. No signs of aggressiveness or increased irritability were observed. The results indicate that the decrease in motor activity induced by large doses of DL 5-HTP is mainly due to an effect outside the blood-brain barrier since the effect is abolished by inhibition of peripheral DC. 5-HTP also appears to have central effects which induce an increased motor activity; these central effects of 5-HTP become obvious only when the peripheral effects of 5-HTP are blocked.

When administered in high doses 5-HTP is taken up and decarboxylated not only in 5-HT neurons but also in CA neurons in the brain. The 5-HT so formed in the CA neurons displaces the stores of transmitters (Fuxe, Butcher and Engel, 1971). Whether this displacement of CA induced by 5-HTP has any functional significance whereby the released CA cause an increased stimulation of central

CA receptors had not been established. Therefore clarification was sought as to whether the stimulant effect of 5-HTP on motor activity obtained after inhibition of peripheral DC is caused predominantly by an exaggerated effect on central 5-HT receptors or by indirect stimulation of central CA receptors.

If the CA neurons were to be pre-empted of CA prior to administration of the 5-HTP (plus MK 486) then per se no CA would be available for release by 5-HT formed from 5-HTP and no stimulation of CA receptors could occur. Treatment with DL- α -methyl metatyrosine (α -MMT) is able to induce a depletion of the stores of CA in CA neurons and causes little sedative effects (Andén 1964, Chan and Webster 1971). It was given to mice in successive injections of 400, 400 and 200 mg/kg 27, 15 and 2.5 h respectively before the administration of DL 5-HTP.

α -MMT given alone induced a marked depletion of DA and NA in brain but had no significant effect on motor activity. The increase in motor activity induced by DL 5-HTP (plus MK-486) was not antagonized by pre-treatment with α -MMT. Thus the fact that 5-HTP (plus MK 486) induced similar stimulatory effects in animals which were already depleted of their stores of CA indicates that the displacement of CA is unlikely to be of importance for this action (see also Rubenson 1971). However the possibility remains that 5-HTP causes the release of small amounts of CA from pools which are not depleted by α -MMT.

The antidepressant drugs chlorimipramine and prtriptyline can selectively block the membrane pump for re-uptake of amines into 5-HT neurons and NA neurons respectively (Carlsson, Corradi, Fuxe and Hökfelt 1969a, b). Consequently chlorimipramine would potentiate effects induced by an increased release of 5-HT since its active re-uptake into 5-HT neurons is blocked whereas prtriptyline would potentiate effects induced by an increased release of NA since its active re-uptake into NA neurons is blocked.

Pretreatment with chlorimipramine markedly potentiated the stimulatory effect of 5-HTP (plus MK 486) on motor activity in mice whereas pretreatment with prtriptyline had no such potentiating effect. In animals pretreated with chlorimipramine (plus MK 486) DL 5-HTP in doses as low as 12.5 mg/kg induced significant increase in the motor activity and maximal increase was obtained after 75 mg/kg; doses higher than 75 mg/kg induced pronounced muscular disturbances. Administration of 5-HTP 75 mg/kg to mice pre-treated with MK 486 caused no significant lowering of CA concentrations in brain.

The fact that chlorimipramine but not protriptyline potentiated the stimulatory effect of 5-HTP on motor activity strongly suggests that this action of chlorimipramine is mediated via its effect on neurotransmission in 5-HT neurons. Thus the increase in motor activity induced by 5-HTP (plus MK 486) would appear to be largely dependent on an increased activation of central 5-HT receptors.

An indirect effect of 5-HTP on CA receptors mediated by displacement of CA from central CA neurons is likely to make little if any contribution to the stimulatory effect of 5-HTP on motor activity since:

1. Pre-treatment with α -MMT to empty the CA neurons of their amine content did not antagonize the stimulatory effect of 5-HTP (plus MK 486).
2. Motor activity in mice which were pretreated with chlorimipramine to block amine re-uptake into 5-HT neurons was markedly increased by a dose of 5-HTP (75 mg/kg after MK 486) producing no significant lowering of the concentrations of CA in brain.
3. If the stimulatory effect induced by DL 5-HTP was largely dependent on an increased release of NA, pretreatment with protriptyline to block amine re-uptake into NA neurons should have potentiated the stimulatory effect but no such potentiation was observed.
4. DL 5-HTP (dose of 12.5 mg/kg given to mice pretreated with chlorimipramine (and MK 486) induced a significant increase in motor activity, whereas a dose of 250 mg/kg of L DOPA (the corresponding precursor in the synthesis of CA) was found by Tränberg (1970) to be required to induce an increase in motor activity in mice pretreated with MK 486. Therefore if the increase in motor activity induced by DL 5-HTP is mediated really by an increased activation of CA receptors, one must assume that DL 5-HTP is far more effective in this respect than equimolar doses of L DOPA.
5. In addition it is noteworthy that no signs of aggressiveness or increased irritability were observed during the experiments. These signs often accompany stimulation induced by drugs which activate CA receptors (vide infra).

Experiments with nialamide (paper III)

Inhibition of MAO results in the accumulation of 5-HT and CA in monoamine neurons and overflow of these monoamines from nerve terminals (Carlsson Dahlström, Fuxe and Lindqvist 1965). Large doses of MAO inhibitors have a psychomotor stimulant effect. These biochemical and behavioural effects seem to be causally related (Carlsson et al 1963; Carlsson et al 1965 for review see Melischer 1966).

The behavioural syndrome induced in mice by inhibiting MAO with nialamide was reported by Corradi (1966) to be antagonized by pretreatment with drugs which block the accumulation of both 5-HT and CA (i.e. DL- α -propylthiophenylcarbamate and L- α -methylthiophenylcarbamate) but not by pretreatment with a drug which selectively blocks the accumulation of CA (p-MPT). Consequently the accumulation of 5-HT in the brain was assumed to be of predominant importance for the nialamide-induced stimulation of psychomotor activity. This view is also supported by findings that the 5-HT precursors L-tryptophan and 5-HTP enhance the behavioural effects of MAO inhibition (Graham-Smith 1971; for review see Monteggia 1962). The availability of p-chlorophenylalanine, a fairly specific inhibitor of tryptophan hydroxylase (Koe and Weissman 1966; Jéquier, Lovenberg and Sjoerdsma 1967) prompted further evaluation of the respective roles of accumulated 5-HT and CA in the stimulant effect of nialamide on psychomotor activity.

Nialamide HCl (200 mg/kg) elicited an initial depression of motor activity in mice followed by an increase in activity which was maximal approximately 4 h after the nialamide injection. The increase in motor activity was initially accompanied by hyperthermia and rapid head movements, and subsequently also by tremor and by hyperextension and abduction of the hind legs. These motor disturbances became pronounced after 4 h with an associated decline in the registered motor activity. Nialamide induced more pronounced accumulation of 5-HT than of DA or NA in brain when measured 3 and 6 h after its injection. Pretreatment with L-tryptophan (300 mg/kg) 1 h before giving nialamide resulted in a more rapid onset of the psychomotor stimulation and faster accumulation of 5-HT than was obtained after nialamide alone. These behavioural and biochemical effects of L-tryptophan are in all probability causally related (cf. Graham-Smith 1971).

PCPA administered alone to mice in either of the two dose regimens mentioned

below was largely without effect on motor activity. It induced a marked decrease in the concentration of 5-HT but not of CA in brain. Pretreatment with PCPA 400 mg/kg 24 h before giving mianserin partially antagonized the increase in motor activity and accumulation of 5-HT in brain which are both induced by mianserin alone. Pretreatment with PCPA 800 mg/kg for 3 consecutive days at 72, 48 and 24 h before giving mianserin completely antagonized the behavioural excitation and hyperthermia and the accumulation of 5-HT which are induced by mianserin alone. However, this latter pretreatment with PCPA also partially antagonized the mianserin-induced accumulation of DA and NA in brain, indicating an impaired synthesis of CA in these animals (Tagliamonte, Tagliamonte, Corsi, Meru and Gessa, 1973).

The additional administration of DL 5-HTP 30 mg/kg 1 h after giving mianserin to the animals twice pretreated with PCPA restored the behavioural excitation. The results indicate that the antagonizing effect of PCPA on the mianserin syndrome is largely due to its inhibitory effect on the synthesis of 5-HT. The inhibitory effect of the triple dose of PCPA on the synthesis of CA may possibly contribute to the complete antagonism of the syndrome. However, the finding that a relatively low dose of 5-HTP completely restored the mianserin-induced syndrome in animals twice pretreated with PCPA suggests that the PCPA-induced inhibition of CA synthesis is not of great importance.

α -MT administered alone to inhibit tyrosine hydroxylase lowered the concentrations of DA and NA in brain. Pretreatment with α -MT 200 mg/kg 2 h before mianserin reduced the mianserin-induced hyperactivity to about the same extent as did pretreatment with PCPA 400 mg/kg and almost completely antagonized the mianserin-induced hyperthermia. Also the accumulation of CA which is induced by mianserin alone was completely blocked by α -MT, indicating an effective inhibition of CA synthesis. The contribution of central CA neurons to the maintenance of normal motor activity is fairly well established (see e.g. Carlsson, Lindqvist and Magnusson, 1957; von Rossum, 1970; Svensson, 1971; Ahlenius, Andén and Engel, 1973) and blockade of the CA synthesis results in depression of motor activity (see e.g. Svensson and Waldeck, 1970). The partial inhibition of the mianserin-induced hyperactivity in animals pretreated with α -MT indicates that the fully-developed mianserin syndrome is at least dependent on functionally intact central CA neurons. The finding that mianserin nevertheless increased the

motor activity in animals pretreated with α -MT emphasizes the role of 5-HT in psychomotor stimulation.

The initial depression of motor activity observed after the administration of nialamide was not antagonized by pretreatment with either PCPA or α -MT. Consequently this behavioural effect seems to be unrelated to the changes in 5-HT and CA concentrations in brain.

In conclusion the hyperactivity induced by nialamide seems to be largely dependent on the inhibition of 5-HT catabolism and hence like the qualitatively similar 5-HTP induced hyperactivity effects on increased activation of central 5-HT receptors. Possibly an increased activation of CA receptors may contribute to some extent. At least the fully developed hyperactivity seems to be dependent on an intact neurotransmission in central CA neurons.

Experiments with L tryptophan (paper IV)

Tryptophan plus metabolite inhibitors

L Tryptophan is metabolized along several pathways both in peripheral tissues and in the CNS (for review see e.g. Guroff and Lovenberg 1970). These include the formation of 5-HT (see Introduction) and of tryptamine (Werle and Mennicken 1937; Haas, Redfield and Udenfriend 1959; Weissbach, King, Sjoerdsma and Udenfriend 1959). A quantitatively important route is the oxidation of tryptophan to liver tryptophan pyrrolase to formylkynurenine in the "kynurenine-pathway" (Greengard and Felgelson 1962; Nemeeth 1962).

L Tryptophan induces an increase in the synthesis of 5-HT in brain which differs from that induced by 5-HTP in two ways: 1) The maximal increase in 5-HT synthesis which can be obtained by administration of L tryptophan is much less pronounced than that which can be obtained by administration of 5-HTP and the tryptophan hydroxylase becomes saturated by lower doses of its substrate than does the DC. The maximal synthesis of 5-HT which could be obtained in mouse brain after administration of L tryptophan equaled 300 ng/kg when given intraperitoneally (Carlsson and Lindqvist 1972). 2) The 5-HT formed in the brain from exogenous L-tryptophan appears to be localized selectively in the 5-HT neurons as result of the specific distribution of tryptophan hydroxylase (see Introduction), whereas when 5-HTP is administered the 5-HT so formed is less specifically localized.

(Id. *supra*) L-Tryptophan is reported to reduce the spontaneous motor activity of laboratory animal (Brown 1960; Ashcraft Eccleston and Crawford 1965) and to induce sedation and changes of the sleep-pattern in man (see Wyatt Engelman Kupfer From Sjoerdsma and Snyder 1970; Hartman Chung and Chien 1971). These two latter effects of L-tryptophan have also been observed in patients pretreated with p-chlorophenylalanine and have consequently been suggested to be unrelated to the increase in 5-HT synthesis induced by L-tryptophan. Further clarification of the central effects of tryptophan on motor activity was therefore sought using inhibitors of the different metabolic pathways for L-tryptophan.

L-Tryptophan (50-800 mg/kg) was administered to mice and their motor activity was recorded 1 h later. Groups of animals were pretreated with drugs which inhibit the above-mentioned metabolic pathways of L-tryptophan. Pretreatment with PCPA (800 mg/kg on three consecutive days) was used to inhibit the tryptophan hydroxylase. MK-486 (75 mg/kg given 30 min before tryptophan) was used to selectively inhibit peripheral DC and NSD-1015 (100 mg/kg given 30 min before tryptophan) was used to inhibit both central and peripheral DC, thus inhibiting the formation of monoamines including tryptamine. Allopurinol (50 mg/kg given 2.5 h before tryptophan) was used as an inhibitor of tryptophan pyrrolase (Becking and Johnson 1967).

Animals given 800 mg/kg of L-tryptophan appeared sedated and their motor activity was significantly reduced. Lower doses of L-tryptophan had no apparent effects on gross behaviour and did not significantly affect the motor activity. None of the various pretreatments antagonised the L-tryptophan-induced reduction in motor activity; pretreatment with PCPA was ineffective, whereas pretreatment with MK-486, NSD-1015 or allopurinol potentiated the behavioural depressant effect of L-tryptophan. I.e. the dose-response curve was shifted to the left in these latter groups of animals. The results indicate that the L-tryptophan-induced reduction in motor activity is not mainly mediated by any of its known neurotropic metabolites, i.e. 5-HT, tryptamine or metabolites along the kynurenine pathway. Possibly therefore the behavioural-depressant effect of L-tryptophan may be mediated by the amino acid itself rather than by any of its metabolites. In view of the sedative effect of L-tryptophan in man it seems likely that its inhibitory effect on motor activity in mice is at least partially of central origin.

Tryptophan plus chlorimipramine:

Chlorimipramine 25 mg/kg was given to mice 30 min before administration of L-tryptophan 50-800 mg/kg. When compared with animals given only chlorimipramine (plus saline) mice given 100 mg/kg L-tryptophan exhibited a moderate increase in motor activity whereas after 800 mg/kg motor activity was reduced and doses of 50, 200 and 400 mg/kg were ineffective.

The hyperactivity induced both by 5-HTP and by l-tryptophan is mediated by 5-HT and is potentiated when re-uptake of transmitter into 5-HT neurons is blocked by chlorimipramine (vide supra). Consequently the administration of 100 mg/kg L-tryptophan may well induce an increased release of 5-HT which in the chlorimipramine pretreated animal may be sufficient to cause an increased activation

of central 5-HT receptors; this being reflected as an increase in motor activity.

Clinical and physiological implications

In the experiments relating to the administration of DL-5-HTP or l-tryptophan to mice a gradual increase in motor activity appeared to be correlated with a gradually increased release of 5-HT from central 5-HT neurons. Hence in these experiments the hyperactivity seems to provide a quantitative measure of the activation of 5-HT receptors. Hyperactivity appeared to result from a marked overstimulation of 5-HT receptors. However the absence of hyperactivity in animals treated with either L-tryptophan or the lower doses of DL-5-HTP with or without pretreatment with MK-486 does not exclude the possibility that the increase obtained in the synthesis of 5-HT after these treatments may have resulted in moderately increased release of 5-HT and activation of 5-HT receptors. The experiments with DL-5-HTP illustrate this: a dose of DL-5-HTP as low as 12.5 mg/kg increased the motor activity of animals pretreated with chlorimipramine plus MK-486 whereas 100 mg/kg was the lowest effective dose of DL-5-HTP in animals pretreated with MK-486 alone. The potentiation is in all probability mainly due to the membrane pump blocking effect of chlorimipramine resulting in an enhanced concentration of 5-HT at the postsynaptic receptors. Thus the results indicate that also when DL-5-HTP 12.5 mg/kg is administered to animals pretreated with MK-486 alone, at least some increase in the release of 5-HT is probably induced.

The increase in motor activity induced by L-tryptophan 100 mg/kg after pretreatment with chlorimipramine suggests for the same reason that this dose of L-tryptophan induces an increased release of 5-HT also when the drug is given alone.

A comparison between the two precursors of 5-HT reveals that DL-5-HTP given after inhibition of peripheral DC has much higher efficacy than L-tryptophan with regard to activation of central 5-HT receptors. Administration of high doses of DL-5-HTP after pretreatment with MK 486 results in pronounced overstimulation of 5-HT receptors. This overstimulation is comparable to that obtained after complete inhibition of MAO. Presumably the inhibition of peripheral DC contributes considerably to the high efficacy of DL-5-HTP (see e.g. Horita and Hamilton 1970 and introduction). Evidence for the increased activation of central 5-HT receptors after administration of L-tryptophan alone has not been presented. However, the lack of evidence may merely reflect the unavailability of sensitive model for estimating the activity of 5-HT receptors. The moderate stimulant effect on behaviour which L-tryptophan 100 mg/kg was found to induce in chlorimipramine-pretreated animals is suggestive evidence for the activation of 5-HT receptors by the 5-HT formed from the administered amino acid. Although this effect is reproducible, the finding requires further study before a definite conclusion can be

The 5-HT formed in CA neurons after administration of high doses of DL-5-HTP did not seem to contribute to the behavioural effects of the precursor. DL-5-HTP would therefore appear to exert its functional effects fairly specifically in 5-HT neurons. In contrast, high doses of L-tryptophan were found to induce behavioural depression by a mechanism unrelated to 5-HT. Furthermore, high doses of L-tryptophan have neuroleptic-like effect on conditioned avoidance behaviour in rats (Engel and Modigh 1974) and induce a partial inhibition of the synthesis of CA in mouse brain (Modigh, unpublished observation). These unspecific effects of L-tryptophan are only obtained after giving doses which are in excess of that required to cause saturation of brain tryptophan hydroxylase. For more accurately interpreting the antidepressant effect of L-tryptophan, there would thus appear to be an urgent need to determine the dose level at which the brain tryptophan hydroxylase is saturated. Tryptophan has been claimed to be a considerably more specific tool with respect to central 5-HT neurons than 5-HTP (see e.g. Mol and Eccleston 1968). The present results do not support this assumption.

The ability of antidepressant drugs to elevate mood is widely assumed to result from a facilitation of neurotransmission in 5-HT neurons. Clinical evaluations of the effects of 5-HTP in the treatment of endogenous depressions are rare and contradictory (vide supra) but in view of the present findings, further study appears to be warranted. DL 5-HTP administered in combination with an inhibitor of peripheral DC alone or together with chlorimipramine seems to act on 5-HT receptors fairly specifically; moreover such treatments are equally as effective as the use of MAO inhibitors. Overdosages of these treatments result in serious intoxications mainly due to overstimulation of 5-HT receptors (vide infra). However the effects of 5-HTP are of shorter duration than those of MAO inhibitors. Therefore the use of 5-HTP in combination with an inhibitor of peripheral DC alone or together with chlorimipramine would appear to be safer than the use of MAO inhibitors when the therapeutic aim is to achieve an effect via activation of central 5-HT receptors.

Overdosage of MAO inhibitors whether given alone or in combination with tricyclic antidepressant drugs results in intoxications which are sometimes lethal (see e.g. Simmons, Carr and Ros 1970, Matthew 1973). The present investigations indicate that 5-HT plays a predominant role in such intoxications as far as example supported by the finding that PCPA blocked both the behavioural excitation and the mortality resulting from complete inhibition of MAO in mice. Hence the administration of drugs which inhibit the synthesis of 5-HT should provide causal therapy for such intoxications.

5-HT in the CNS was originally proposed on the basis of results of experiments with reserpine to serve as a modulator of a trophotropic system which when activated would cause behavioural depression and sleep (Brodi and Shor 1957, Brodi, Corser, Costa and Diabac 1966, Brodi and Reid 1968). Considerable evidence now indicates that the sedation induced by reserpine is due mainly to an impaired neurotransmission in central CA neurons and is largely unrelated to effects on the 5-HT neurons (see e.g. Carlsson et al 1957, Carlsson 1963, Butcher, Rhodes and Yurwiler 1972). The results of more recent studies of behavioural effects

led to an impaired neurotransmission in 5-HT neurons are in agreement with the original hypothesis proposed by Brodi and co-workers: pharmacological blockade of the 5-HT neurons leads to an increased reactivity to external stimuli (see e.g. Koe and Weissman 1966, Brady Jr 1970, Butcher and Di Leitch 1973) and to

Insomnia (for review see Jouvet 1973). Moreover, lesion of raphe neurons results in behavioural excitation (Kostowski, Giacalone, Garattini and Valzelli 1968) and insomnia (see Jouvet 1973). However, electrical stimulation of raphe cells does not induce sedation (Aghajanian and Sheard 1968). Furthermore, in all the present experiments in which an increased activity of 5-HT receptors was induced no behavioural depression was observed which could be related to an increased activation of 5-HT receptors. Hence there is evidence in support of the view that central 5-HT neurons are involved in the integration of external stimuli and in the regulation of sleep. However, 5-HT does not seem to act as an inhibitory modulator. In the sense that physiologically increased activity of the 5-HT neurons would be expected to result in sedation.

STUDIES ON INTERRELATIONS BETWEEN NERVOUS ACTIVITY AND SYNTHESIS OF TRANSMITTER IN 5-HT NEURONS

Experiments with chlorimipramine and PCPA (papers V and VI)

The administration of chlorimipramine or related inhibitors of the membrane pump of 5-HT neurons suppresses the impulse activity in 5-HT neurons (Sheard et al 1972; see also Corradi and Fuxe 1968) and partially inhibits the synthesis of 5-HT (Meek and Werdinius 1970; Schubert, Nybäck and Sedvall 1970; Schildkraut, Schonberg, Breese and Kapin 1969; Bruinvel 1972). The suppression of impulse activity seems likely to be a feed-back phenomenon which is elicited by the increased activation of pre- or postsynaptic 5-HT receptors following the inhibition of amine re-uptake (vide supra). The activity of brain tryptophan hydroxylase appears from lesion and stimulation experiments to be partially dependent on the impulse activity of 5-HT neurons (see Introduction). Hence the inhibitory effect of chlorimipramine and related drugs on the synthesis of 5-HT may be secondary to that inhibitory effect on the impulse activity as has been suggested by Schubert et al (1970). Alternatively these drugs may directly inhibit the tryptophan hydroxylase or the uptake of tryptophan into the 5-HT neurons. The latter possibility has been emphasized by Bruinvel (1972) who found that imipramine competitively inhibits the uptake of L-tryptophan into synaptosomes.

A possible relation between inhibition of the nerve impulse activity and inhibition of the 5-HT-synthesis after administration of chlorimipramine was investigated by studying the effects of the drug on the rate of tryptophan hydroxylation in the rat spinal cord above and below thoracic transection (see Methodology). For comparison the effects of PCPA which directly inhibits tryptophan hydroxylase (vide supra) were studied in parallel. The rate of synthesis of 5-HT was estimated in one of two ways: 1) in the experiments using chlorimipramine the accumulation of 5-HTP 20 min after administration of NSD 1015 100 mg/kg referred to as 5-HTP (after NSD 1015) was measured; 2) when using PCPA the accumulation of 5-HT 2 1/2 and 5 h after administration of l-tyrosine HCl 500 mg/kg referred to as 5-HT (after l-tyrosine) was measured. After an acute spinal transection the accumulation of 5-HTP (after NSD 1015) was reduced by approximately 40 per cent in the spinal cord below the transection but was not significantly effected in the cranial part. By contrast the acute transection did not signifi-

cantly affect the accumulation of 5-HT (after nialamide) in either of the two halves of the spinal cord. These results are in agreement with earlier reports (Carlsson et al. 1973b; Meek and Fuxe 1971). The reduced accumulation of 5-HTP (after NSD 1015) below a spinal transection indicates that the synthesis of 5-HT was decreased in the caudal part, probably due to blockade of the nerve impulse flow.

Administration of MAO inhibitors soon leads to a partial inhibition of the 5-HT synthesis (Mason et al. 1971; Carlsson, Bédard, Davi, Kehr, Lindqvist and Magnusson 1973a) and to an almost complete inhibition of the impulse activity in the 5-HT neurons (Aghajanian, Graham and Sheard 1970). The difference in impulse activity in 5-HT neurons above and below spinal transection is therefore largely abolished shortly after the administration of nialamide. Thus the finding that the accumulation of 5-HT (after nialamide) is unaffected by spinal transection supports the view that the reduced accumulation of 5-HTP (after NSD 1015) below an acute spinal transection is due to blockade of the impulse flow in the 5-HT neurons and not to other consequences of the transection.

When PCPA (400 mg/kg) was administered to rats 1 h after spinal transection, then 24 h later the 5-HT synthesis (5-HT accumulation after nialamide) was found to be equally inhibited both above and below the transection.

Chlorimipramine (15 mg/kg) administered 2 h after a spinal transection reduced the 5-HT synthesis (5-HTP accumulation after NSD 1015) in the brain and in the rostral part of the spinal cord, but below the transection the 5-HT synthesis was not significantly affected. After the chlorimipramine, the concentration of tryptophan in the CNS was not changed in either intact or transected animals.

The different effects of PCPA and chlorimipramine on the rate of 5-HT synthesis in the spinal cord below transection indicate that the two drugs must inhibit the 5-HT synthesis by different mechanisms. The ineffectiveness of chlorimipramine below spinal transection is in keeping with the view that the drug inhibits the 5-HT synthesis by depression of the nerve impulse activity.

Reserpine (10 mg/kg) given 4 h before chlorimipramine (15 mg/kg) was found to prevent the inhibitory effect of chlorimipramine on the accumulation of 5-HTP (after NSD 1015) (table 1); reserpine itself is reported not to significantly affect the accumulation of 5-HTP (Carlsson and Lindqvist 1972; compare also the control groups in table 1 and fig. 1). In contrast, PCPA given to reserpine-pretreated animals effectively inhibits the 5-HT synthesis (5-HT accumulation after nialamide)

Table 1. Concentrations of 5-HTP and tryptophan in rat brain 30 min after the administration of NSD 1015 100 mg/kg. The animals were pretreated with reserpine 10 mg/kg 1-4 1/2 h and either chlorimipramine 15 mg/kg or 9% w/v NaCl at 30 min before the administration of NSD 1015. The drugs were administered by the intraperitoneal route. Each determination of 5-HTP and tryptophan was made from single whole brain. Each value represents the mean of 7 determinations \pm s.e.

Pretreatment before NSD 1015	5-HTP $\mu\text{g/g}$	Tryptophan $\mu\text{g/g}$
Reserpine + 9% saline	0.097 ± 0.007	5.69 ± 0.08
Reserpine + chlorimipramine	0.110 ± 0.012	5.63 ± 0.09

(Koe and Weissman, 1966). The main effect of reserpine is to block the function of the storage granules in monoamine neurons (for review see Carlsson, 1965) which in turn nullifies the ability of the nerve impulses to release the monoaminergic transmitters. Therefore, after pretreatment with reserpine, the blockade of the membrane pump induced by chlorimipramine may be assumed to be functionally ineffective at the 5-HT receptors. Furthermore, reserpine is reported to partially depress the impulse activity of 5-HT neurons (Aghajanian and Holzger, 1973). Consequently, the antagonizing effect of reserpine on the inhibition of the 5-HT synthesis induced by chlorimipramine is in agreement with the feed-back hypothesis mentioned above.

Chlorimipramine given in various doses (3.75-60 mg/kg) was studied for its effects on the rate of synthesis of 5-HT (accumulation of 5-HTP after NSD 1015) in brain and cranial and caudal spinal cord of unoperated rats (fig. 1). Each dose of chlorimipramine gave similar effect in the three parts of the CNS. 3.75 mg/kg of chlorimipramine had no significant effect, whereas doses between 7.5 and 60 mg/kg were almost equally effective in reducing the accumulation of 5-HTP by approximately 35 per cent. This reduction is of the same magnitude as that obtained in the caudal part of the spinal cord after an acute midthoracic transection (vide supra). Interestingly, the smallest effective dose in the present experiments is almost identical to the ED₅₀ (intraperitoneal administration in mice)

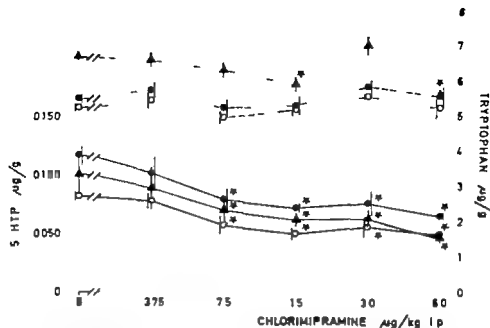


Fig 1 Effects of various doses of chlorimipramine given 30 min before NSD 1015 100 mg/kg on the concentration of 5-HTP (—) and tryptophan (---) in the brain (▲) and in the cranial (○) and caudal parts (●) of the spinal cord of unanesthetized rats. The drugs were administered by the intraperitoneal route. The animals were killed 30 min after the injection of NSD 1015. For each determination of 5-HTP and tryptophan, one whole brain or four pooled halves of the spinal cord were used. Each point represents the mean of 6 determinations \pm s.e.m. The data were evaluated statistically by means of analysis of variance followed by t-test (Winer 1962). ★ represents $p < 0.05$ in comparison to control group given 9% saline solution plus NSD 1015 (=chlorimipramine 0 mg/kg).

for the chlorimipramine-induced blockade of the membrane pump in 5-HT neurons (Carlsson 1970). If chlorimipramine inhibited the 5-HT synthesis mainly by competitively inhibiting the uptake of tryptophan into 5-HT neurons, as was suggested for Imipramine (Idé supra), then the inhibition of the accumulation of 5-HTP would have been expected to be increasingly effective after doses of chlorimipramine higher than 7.5 mg/kg.

The concentration of tryptophan in the spinal cord was not significantly changed by any of the doses of chlorimipramine (Fig. 1). The brain concentration

of tryptophan was slightly but significantly reduced by chlorimipramine in the doses 15 and 60 mg/kg whereas the other doses had no significant effects. Some of the animals given the highest dose of chlorimipramine (60 mg/kg) showed toxic reactions. The largely unchanged concentrations of tryptophan found after chlorimipramine suggests that the drug in the dose range investigated has little if any effect on the uptake of tryptophan into the CNS.

In conclusion the present experiments show that PCPA and chlorimipramine inhibit the synthesis of 5-HT by different mechanisms the inhibition induced by chlorimipramine probably being mediated mainly via inhibition of the impulse flow in 5-HT neurons. Consequently the results also give additional support to the view that the 5-HT synthesis is to some extent regulated by the nerve impulse activity.

PCPA

PCPA inhibits the brain tryptophan hydroxylase both *in vivo* and *in vitro*. The inhibition of the enzyme *in vivo* becomes irreversible after an initially reversible phase (Koe and Wilson 1966; Jéquier *et al.* 1967) but the mechanism of the irreversible inhibition has not been completely clarified (for review see Koe 1971). Gél and co-workers have shown that PCPA is incorporated into different enzyme proteins including phenylalanine 4-hydroxylase in liver and proteins with tryptophan hydroxylase activity in brain. They postulated that the irreversible inhibition of tryptophan hydroxylase by PCPA is due to its incorporation into newly built enzyme protein at or near to the active site (Gél, Røsgaarden and Millard 1970; Gél and Millard 1971). However in the present experiments the inhibition of 5-HT synthesis (accumulation of 5-HT after nialamide) was the same whether PCPA was given to animals spinally transected 1 h earlier or to intact animals. A spinal transection blocks the supply of newly-formed tryptophan hydroxylase to the spinal cord below the transection (see Methodology). Therefore the result indicates that 24 h after the administration of PCPA the inhibition of 5-HT synthesis is not dependent on the incorporation of PCPA into the newly-formed enzyme. This inhibition is probably mainly irreversible (see Jéquier *et al.* 1967). The results also indicate a slow turnover of tryptophan hydroxylase in the spinal cord which is in agreement with report by Meek and Neff (1972).

Hence the differences in the effect of fighting on 5-HT depletion and on 5-HTP accumulation may to some extent reflect possible variations in stress intensity. A more likely explanation for the different effects of fighting on 5-HT depletion and on 5-HTP accumulation is that the rate of 5-HT synthesis increases as a result of the increase in concentration of brain tryptophan (Tagliamonte, Tagliamonte, Perz-Cruet, Stern and Gessa 1971; Carlsson and Lindqvist 1972; Curzon, Joseph and Knott 1972) whereas the impulse flow in the 5-HT neurons is largely unchanged. Therefore the increase in 5-HT metabolism during fighting is unlikely to reflect correspondingly increased activation of postsynaptic 5-HT receptors.

Effects of isolation and fighting on CA in brain

The synthesis and release of the CA in brain during the isolation and fighting experiments were studied in parallel with the 5-HT synthesis was measured by determining the rate of accumulation of DOPA after administration of NSD 1015 (100 mg/kg) and release by determining the rate of depletion of DA and NA after administration of α -MT (250 mg/kg) (see Methodological considerations). In brief DOPA accumulated at a lower rate in "isolated" than in "grouped" animals and the accumulation was markedly accelerated during fighting. Since these differences in DOPA accumulation were equally pronounced in DA- and NA-rich areas of the brain they probably reflect differences in the rates of synthesis of both DA and NA. The depletion of DA and NA induced by α -MT was slower in isolated (see Welch and Welch 1968b) than in grouped animals and was accelerated during fighting. The results indicate that the impulse flow and rate of synthesis of transmitter in DA- and NA neurons in brain are retarded during isolation but are rapidly accelerated during fighting. The rate of synthesis of CA is generally considered to be regulated mainly by the impulse flow in the CA neurons (for review see Andén et al. 1969a). Hence the observed differences in CA synthesis are likely to be secondary to the differences in impulse flow. Central NA and DA neurons have both been proposed to be involved in the expression of aggressive behaviour (see e.g. Fog 1969; Randrup and Munkvad 1969; Welch and Welch 1969; McKenzie 1971). The present result supports this view.

Clinical and physiological implications

Variations in the rate of 5-HT synthesis which are related to changes of the impulse flow in 5-HT neurons appear from experiments using stimulation and lesions to be due to variations in activity of the tryptophan hydroxylase rather than to variations in substrate concentration near the enzyme (Gibbels and Eklund 1972; Curzon et al. 1972). Curzon and co-workers have suggested that the activity in 5-HT neurons may also influence mechanisms which determine the concentration of brain tryptophan. i.e. an increased demand for 5-HT would induce a feed-back mechanism to induce an increased concentration of tryptophan in the brain. This hypothesis is based on the finding of a statistically significant correlation between the increased concentrations of tryptophan and 5-HIAA in brain during stress (Curzon et al. 1972; Knott, Joseph and Curzon 1973). Similar biochemical effects were found in the present experiments when studying the stress of fighting. However, these effects did not seem to be accompanied by an increased impulse flow in the 5-HT neurons. Hence, at least during the stress of fighting, changes in the 5-HT synthesis may occur which are unrelated to the impulse activity in 5-HT neurons.

In view of the above considerations, the functional activity in 5-HT neurons is probably more reliably reflected by estimating the tryptophan hydroxylase activity than by estimation of the total turnover of 5-HT. At present, in clinical studies of the possible involvement of 5-HT in mental diseases, estimations of the total turnover of 5-HT in the CNS are made from measurements of the accumulation of 5-HIAA in samples of cerebrospinal fluid (CSF) after probenecid treatment (see e.g. Roos and Sjostrom 1969; Sjostrom and Roos 1972). No clinically applicable method for estimating the activity of brain tryptophan hydroxylase in man has yet been developed. Possibly, the ratio between the concentrations of tryptophan and 5-HIAA in the CSF may be an index of the activity of this enzyme. If such a relationship could be demonstrated experimentally in laboratory animals, then the concomitant determination of 5-HIAA and tryptophan in human CSF should provide a means of estimating brain tryptophan hydroxylase in man. This in turn would probably more accurately reflect the functional activity in 5-HT neurons.

Hence the differences in the effect of fighting on 5-HT depletion and on 5-HTP accumulation may to some extent reflect possible alterations in stress intensity. A more likely explanation for the different effects of fighting on 5-HT depletion and on 5-HTP accumulation is that the rate of 5-HT synthesis increases as a result of the increase in concentration of brain tryptophan (Tagliamonte, Tagliamonte, Perez, Crust, Stern and Gessa, 1971; Carlsson and Lindqvist, 1972; Curzon, Joseph and Knott, 1972) whereas the impulse flow in the 5-HT neurons is largely unchanged. Therefore the increase in 5-HT metabolism during fighting is unlikely to reflect correspondingly increased activation of postsynaptic 5-HT receptors.

Effects of Isolation and fighting on CA in brain

The synthesis and release of the CA in brain during the isolation and fighting experiments were studied in parallel with the 5-HT synthesis was measured by determining the rate of accumulation of DOPA after administration of NSD 1015 (100 mg/kg) and release by determining the rate of depletion of DA and NA after administration of α -MT (250 mg/kg) (see Methodological considerations). In brief DOPA accumulated at lower rate in isolated than in grouped animals and this accumulation was markedly accelerated during fighting. Since these differences in DOPA accumulation were equally pronounced in DA- and NA-rich areas of the brain they probably reflect differences in the rates of synthesis of both DA and NA. The depletion of DA and NA induced by α -MT was lower in isolated (see Welch and Welch, 1968b) than in grouped animals and was accelerated during fighting. The results indicate that the impulse flow and rate of synthesis of transmitter in DA- and NA neurons in brain are retarded during isolation but are rapidly accelerated during fighting. The rate of synthesis of CA is generally considered to be regulated mainly by the impulse flow in the CA neurons (for review see Andén et al., 1969a). Hence the observed differences in CA synthesis are likely to be secondary to the differences in impulse flow. Central NA and DA neurons have both been proposed to be involved in the expression of aggressive behaviour (see e.g. Fog, 1969; Randrup and Munkvad, 1969; Welch and Welch, 1969; McKenzie, 1971). The present results support this view.

the 5-HT formed in 5-HT neurons or in the 5-HT formed in CA neurons. Pretreatment with DL α -methyl-metatyrosine in a dose which effectively depleted the stores of CA in brain did not counteract the stimulant effect of MK 486 plus DL 5-HTP on motor activity. Pretreatment with chlorimipramine in a dose which inhibits fully specifically the re-uptake of transmitter in 5-HT neurons markedly potentiated the psychomotor stimulant effect of MK 486 plus DL 5-HTP. Protriptyline administered in a dose which inhibits the re-uptake of transmitter in NA neurons but not in 5-HT neurons had no such potentiating effect. The results indicate that the hyperactivity induced by MK 486 plus DL 5-HTP is mainly due to an increased formation of 5-HT in and release of the transmitter from 5-HT neurons. The 5-HT formed in CA neurons in brain after administration of DL 5-HTP does not appear to induce any marked effects on the function of these neurons. In conclusion DL 5-HTP administered after inhibition of peripheral DC appears to be as effective as nialamide for inducing stimulation of 5-HT receptors in the brain and 5-HTP exerts its functional effects relatively specifically in the 5-HT neurons.

3 L-tryptophan when administered in a dose in excess of that required to saturate brain tryptophan hydroxylase was found to reduce the motor activity in mice. Doses of L-tryptophan which do not cause saturation of the enzyme induced no apparent behavioural effects. Pretreatment with drugs inhibiting either tryptophan hydroxylase (i.e. PCPA), peripheral DC (i.e. MK 486) or both peripheral and central DC (i.e. NSD 1015) or pyrazole (i.e. allopurinol) did not antagonise the behavioural depression induced by large doses of L-tryptophan. On the contrary several of these pretreatments potentiated the behavioural depressant effect of L-tryptophan. The results indicate that the behavioural depression is not primarily mediated by the neurotropic metabolites of L-tryptophan in 5-HT tryptamine or metabolites along the kynurenine pathway. Animals pretreated with chlorimipramine showed a slight but statistically significant increase in motor activity after administering L-tryptophan in a dose somewhat lower than that required to saturate brain tryptophan hydroxylase. This finding is suggestive evidence for an increased activation of central 5-HT receptors by 5-HT formed from administered L-tryptophan. In conclusion the results indicate that administration of L-tryptophan gives rise to an increased activation of central 5-HT receptors.

L Tryptophan has however a much lower efficacy in this respect than does DL 5-HTP administered after inhibition of peripheral DC. Furthermore high doses of L tryptophan induce behavioural effects by mechanisms unrelated to central 5-HT neurons.

4 No signs of behavioural depression relating to an increased activation of central 5-HT receptors were found in any of the experiments described above. On the contrary treatment with drugs which increased the stimulation of central 5-HT receptors induced behavioural excitation. Thus the present results are in opposition to the concept that 5-HT acts as an inhibitory modulator in the sense that physiologically-increased activity in the 5-HT neurons would be expected to result in sedation.

5 Chlorimipramine has been previously reported to lower the rate of synthesis of 5-HT and to almost completely suppress the firing of raphe neurons. The suppression of the nerve impulse activity is likely to be a feed-back phenomenon elicited by an increased activation of pre- or postsynaptic receptors after inhibition of the re-uptake of transmitter in the 5-HT neurons. Attempts were made to

test whether the reduction in 5-HT synthesis obtained after administration of imipramine is an effect secondary to the suppression of the impulse activity of 5-HT neurons or is due to a direct inhibitory effect on 5-HT synthesis. Administration of chlorimipramine to rats reduced the rate of tryptophan hydroxylation which was estimated in brain and spinal cord tissue by the accumulation of 5-HTP after inhibition of DC. A midthoracic transection of the spinal cord blocks the impulse flow in 5-HT neurons below the transection. In agreement with previous reports after an acute transection of the spinal cord the rate of tryptophan hydroxylation was found to be reduced. Chlorimipramine reduced the rate of tryptophan hydroxylation above but not below the acute transection. PCPA was by contrast found to equally inhibit the 5-HT synthesis above and below the transection. Pre-treatment with reserpine completely antagonized the inhibitory effect of chlorimipramine on the rate of tryptophan hydroxylation. A dose-response study revealed that the magnitude of the reduction in tryptophan hydroxylation induced by the lowest effective dose of chlorimipramine was of the same order as that induced by a sevenfold higher dose and was also of the same magnitude as the reduction in tryptophan

hydroxylation induced by an acute transection of the spinal cord. The concentration of tryptophan in the CNS was largely unaffected by the various doses of chlorimipramine. Taken together, these results suggest that the reduction in the synthesis of 5-HT obtained after administration of chlorimipramine is mediated mainly by inhibition of the impulse flow in 5-HT neurons.

6. A variety of physical and psychological forms of stress have previously been reported to increase the synthesis of 5-HT and the concentration of tryptophan in the brain. Attempts were made to clarify whether these biochemical effects of stress are related to the impulse activity in 5-HT neurons. Male mice were isolated for several weeks in order to induce intensive fighting when brought together in groups of 20-30. The activity in 5-HT neurons and the rate of tryptophan hydroxylation in the brains of such fighting animals were estimated by determining the rate of depletion of 5-HT after inhibition of tryptophan hydroxylase and the rate of accumulation of 5-HTP after DC inhibition, respectively. The concentration of tryptophan and the accumulation of 5-HTP in brain were higher in the fighting animal than in control animals which remained isolated. The rate of depletion of 5-HT after inhibition of synthesis was, however, almost identical in fighting and isolated animals. In conclusion, the increased synthesis of 5-HT occurring during fighting is probably secondary to the increase in concentration of tryptophan in brain and occurs independently of the impulse activity in 5-HT neurons.

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Supplementum 404

A SPECTROPHOTOMETRIC METHOD
FOR ANALYSIS OF OXYGEN CONSUMPTION
IN VITRO ON THE MICROSCALE

BY

RAGNAR HULTBORN



ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 404

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This thesis is partly based on the following publications

- 1 Ragnar Hultborn A sensitive method for measuring oxygen consumption *Anal. Biochem.* 47 442–450 1972
- 2 Hans Herlitz and Ragnar Hultborn A microspectrophotometric technique for determination of respiration in comparison to the Cartesian diver method. — Respiratory activity of rat corpus luteum cells with reference to substrate *Acta physiol. scand.* In press.
- 3 Ragnar Hultborn and Holger Hydén Microspectrophotometric determination of nerve cell respiration at high potassium concentration. *Exp. Cell Res.* submitted for publication
- 4 Ragnar Hultborn, Sune Larsson and Lennart Pålhage An automatic cuvet changer for spectrophotometric oxygen consumption measurements and for conventional spectrophotometry *Zeiss Informationen* accepted for publication
- 5 Ragnar Hultborn and Sante Olling Studies on leucocyte function by measuring respiration and nitroblue tetrazolium reduction by simplified methods *Scand. J. Clin. Lab. Invest.* 32, 297–304 1973

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Introduction

Metabolism is fundamental for cell life and is dependent on a chain of enzymic processes, the energy required mainly being supplied by mitochondrial oxidative activity. Hence analysis of oxygen consumption constitutes an important approach to studies of how enzymic reactions are affected in normal or abnormal cells by changes in their functional activity as well as in their chemical or physical environment. It must be recognized that results from investigations on gas exchanges represent a cellularly integrated all-over measure of oxidative activity though intermediate reactions may be studied using specific substrates or metabolic inhibitors. This integrated functional activity often considerably differs from the potential activity of a cell as indicated by its enzyme and metabolite content.

Due to cellular heterogeneity of almost any tissue metabolic studies must often be performed on single cells or on a tiny tissue sample. In such cases it is a prerequisite to have access to sufficiently sensitive methods. The present work was designed to evolve a sensitive convenient method capable of measuring oxygen consumptions ranging from $1 \mu\text{l/h}$ to $10^{-6} \mu\text{l/h}$. The principle of the technique is to incubate cells or tissue samples in oxyhemoglobin solutions in minute cuvettes. The absorbance shift of the incubate during hemoglobin reduction is then recorded spectrophotometrically.

Manometric methods for estimating exchange of gases on the macroscale have been used for a long time a wide variety of techniques being employed. One type of respirometer which has met with wide use is commonly called the "Warburg" instrument, although as pointed out by Warburg (1926) it was used before his adoption of it. The respirometer is based on the principle that at constant temperature and gas volume any change in amount of gas can be measured by changes in its pressure. A detailed review of manometric procedures for macroscale investigations is given in Umbreit *et al.* (1972). Several micro-adaptations of manometric procedures have been presented, though the Cartesian diver principle adopted by Linderstrom-Lang (1937) proved to be the one most commonly used. Adaptations of this procedure have led to the possibility of analyses of gas exchange in the range of 10^{-4} to $10^{-8} \mu\text{l/h}$ (*cf.* Holter and Zeuthen 1966). Recently Ornan and Brzin (1977) published a modification of the Cartesian diver principle in essence not manometric where the authors claim to have a sensitivity of $10^{-6} \mu\text{l/h}$.

Though the theory of polarographic analysis of oxygen tension was published some 50 years ago (Heyrovsky 1923) it remained essentially unused until the past decade (*cf.* Lessler and Brierty 1969). Polarographic instruments have now been modified for microscale work and are widely employed for determinations of oxygen tension *in vivo* (Payne and Hill 1966, Libbers 1969). Oxygen electrodes are however not suited for O_2 -uptake studies on a single cell level since technical difficulties would arise due to the inevitably minute incubation chambers. Furthermore the electrodes have an intrinsic consumption of oxygen which might be of significance on this scale of work.

The advent of spectrophotometry in the early nineteenth century soon led to the application of this technique in the field of biological sciences. Simultaneously with the increasing knowledge of the spectral characteristics of hemoglobin and studies on tissue

metabolism, Vierordt as early as 1875 made the observation that the spectrum of reflected light from the skin of the hand changed from having two absorbing bands in the visible light to only one broader band upon occlusion of the blood supply to the hand. Vierordt explained this phenomenon correctly as being the expression of tissue metabolism, reducing the oxyhemoglobin within the skin. Vierordt (1878) and his pupil Dennig (1883) further reported on this reaction but the observations were then forgotten for almost half a century when Kahn (1922) further developed these *in vivo* investigations. Since then a fairly large number of reports on tissue metabolism *in vivo* studied by either reflection or transmission photometry have appeared (e.g. Meyer and Reinhold 1926 Koch 1928 Nicolai 1937 Wodick and Lübbert 1973). During the same period an ever increasing number of investigations on spectrophotometric determination of hemoglobin saturation of blood appeared (cf Nilsson 1960) procedures which to a large extent replaced the laborious gas extraction techniques (e.g. Van Slyke 1924). Spectrophotometric approaches for metabolic investigations *in vitro* using hemoglobin as indicator of oxygen tension have only recently been reported. Arvanitaki and Chalazonitis (1949) found a hemoprotein situated intracellularly in the giant neurons of *Aplysia* and *Helix*. The degree of oxygen saturation of this hemoglobin compound could be recorded microspectrophotometrically and be related to the O_2 tension of the environment (cf Chalazonitis 1968) Bärzu and Borza (1967) Bärzu *et al.* (1968) Bärzu and Satre (1970) and Bärzu *et al.* (1972) described in a series of excellent investigations quantitative measurements of oxidative activity of mitochondrial suspensions in "standard" cuvettes containing diluted oxyhemoglobin solutions. The use of cytochromes in oxidation reduction experiments especially fast reactions have been studied by e.g. Chance (cf 1965) though these investigations did not aim at oxidative O_2 uptake determinations.

Theory of the in vitro spectrophotorespirometric system

The microrespirometric procedure described in this work is based on spectrophotometry, recording of the absorbance change of an oxyhemoglobin solution when reduced due to the presence of respiring material within a sealed cuvet. The aim of the methodological development was to design a procedure having a large sensitivity range especially extending so far in the microscale that the respiratory activity of single small cells could be measured. This demanded use of extremely small cuvetts, also referred to as incubation chambers, to decrease the amount of available oxygen, in physical solution as well as bound to the hemoglobin. A decrease in amount of O_2 could also be accomplished by use of low pigment (*i.e.* hemoglobin) concentrations.

Several physico-chemical relationships determining the functional properties of such a system must be considered for correct interpretation of the results. Below a discussion of the most important ones is presented.

Absorbance characteristics of hemoglobin solutions.

Not long after the introduction of spectrophotometry the technique was used in the study of hemoglobin. In 1862 Hoppe published his observations of absorption bands from oxyhemoglobin in the green and yellow part of the spectrum. Stokes (1864) recognized spectrophotometrically the reversibility of oxygenated and reduced hemoglobin. In 1873 Vierordt and in 1880 MacMunn published extensive monographs on the use of spectroscopes, the latter dealing primarily with the absorption spectra of hemoglobin and its numerous derivatives. Soret (1878-1883), Arsonvalles (1890) and Gemgee (1897) were the first to report the spectral characteristics in the blue-violet and ultra-violet regions. During this century an ever increasing amount of literature on the absorption spectra of hemoglobin and its derivatives has appeared and recently Assendelft (1970) published a detailed monograph from which extinction data used in this work have been obtained (Fig. 1).

In the respirometric system considered here measurements are made on solutions of oxygenated and deoxygenated hemoglobin in various proportions: the solutions generally being rather dilute compared to whole blood (dilution 1/20 to 1/50), and the optical pathlengths being short (1 mm to 0.1 mm). To obtain sufficient absorption it is necessary to select a wavelength where the extinction coefficients are high, *i.e.* it is not advantageous to choose the red part of the spectrum generally used for analyses of O_2 saturation in whole blood (*in vivo* or *in vitro*). Measurements in the blue region however yield sufficiently low transmission for the present purpose. Another requirement is that a region of the spectrum be selected where the extinction coefficient of reduced and oxygenated hemoglobin differ preferably to a large extent. A wavelength region fulfilling the above mentioned demands exists at 435 nm. In this region the slopes of the two extinction curves ($\frac{d\epsilon}{d\lambda}$)₄₃₅ are similar (Fig. 1) thus a change in pathlength of the monochromatic light used (see below) will not drastically influence the amplitude

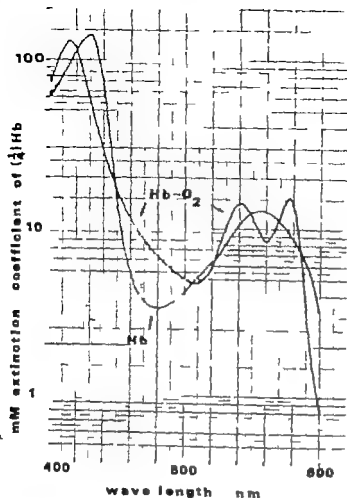


Fig. 1 Extinction-wavelength curves for oxygenated and reduced hemoglobin. Any wavelength region where there is a great difference between extinction coefficients of the two types of hemoglobin can be used for recording of absorbance change during pigment reduction. Molar extinction coefficients for one heme and globin moiety (1/4) Hb are given. (Data obtained from Arsendelli 1970)

the measured difference in absorption, i.e. the change in optical density (OD) between oxygenated and reduced hemoglobin. The relative difference in absorption at 435 nm (2.5 times) is exceeded only in the red part of the spectrum. Extinction coefficients of oxygenated, $\epsilon(1/4)\text{HbO}_2$ and reduced hemoglobin $\epsilon(1/4)\text{Hb}$ at 435 nm, is 41×10^3 and 115×10^3 respectively (Fig. 1)

Oxygen-hemoglobin dissociation kinetics.

The complex nature of the reversible binding of oxygen to hemoglobin was first reported by Bohr (1904) who noticed a non-linearity in the equilibrium $\text{Hb}_4 + 4\text{O}_2 \rightleftharpoons (\text{HbO}_2)_4$. The sigmoid relationships between hemoglobin saturation and oxygen tension was mathematically analysed by Hill (1910) Adair (1925) and others and today the complex molecular interactions during oxygen binding are under intense investigation (cf Rorth and Astrup 1972, Rorth 1973). Several different approaches to the experimental determination of the oxygen dissociation curve have been devised. Discontinuous recording of this curve was done by tonometry (letting the solution come in equilibrium

with a gas mixture of known composition) followed by gasometric determination of the oxygen content in the solution (e.g. Roughton 1963). Discontinuous tonometry followed by spectrophotometric determination of the hemoglobin saturation was also performed (e.g. Benesch *et al.* 1965). During the past two decades continuous recordings of the oxygen dissociation curve have been made by use of spectrophotometry in combination with a gradual change of the gas mixture with which the solution is in equilibrium (e.g. Sick and Gersonde 1969). Similarly the simultaneous use of oxygen electrodes and spectrophotometry combined with an oxygen consuming and producing system which changes the O_2 tension in the solution has been introduced (Colman and Longmuir 1963, Longmuir and Chow 1970).

It was early observed that the equilibrium between oxygen and hemoglobin was influenced by various physico-chemical factors such as hemoglobin concentration, temperature, pH, carbon dioxide and several organic compounds. During the past decade an immense interest has been focussed on the action of the organic phosphate compounds 2,3-diphosphoglycerate and inositol hexaphosphate (phytic acid) since these physiologically occurring substances (the former in mammals, the latter in birds) drastically decrease the oxygen affinity of hemoglobin (Benesch and Benesch 1967, Chanutin and Cumlish 1967, Benesch *et al.* 1968, Gibson and Gray 1970, *cf.* Kulmartin and Rossi-Bernardi 1973).

The most important characteristics of the dissociation kinetics of a hemoglobin solution is the half-saturation value (P_{50}) i.e. the oxygen tension at which 50% of the hemoglobin is oxygenated and the slope of the dissociation curve at its linear part. This slope can be expressed in various ways and in this work it is denoted by the change in hemoglobin saturation ($z \text{ HbO}_2$) per unit change in oxygen tension $\frac{\Delta z \text{ HbO}_2}{\Delta P_{O_2}}$. *In vivo*

P_{50} is generally between 20 and 30 mm Hg and the slope is $\sim 0.02 \text{ mm Hg}^{-1}$. In the *in vitro* system presented in this work it will be noticed that the P_{50} value is decreased and the slope is increased due to hemoglobin dilution and (partial) "stripping" of hemoglobin from its phosphate compounds (Berman *et al.* 1971). The half saturation value is of importance when considering the environment in which cellular respiration is measured in the procedure described in this work (see General Discussion). Further it is necessary to know the slope of the dissociation curve for the calculation of oxygen uptake in the system (see below).

The O_2 -content in a closed system as related to oxygen tension, time and hemoglobin concentration.

The oxygen present in a hemoglobin solution is divided into two pools, one physically dissolved and the other bound to the hemoglobin. The relative amounts of these pools are dependent on the pigment concentration. When this is low the former pool will dominate, when high the latter.

The amount of oxygen in physical solution is directly proportional to the oxygen tension, but the pool combined with the hemoglobin is not, as discussed above. The total amount of oxygen in the chamber is thus nonlinear with regard to O_2 tension since it is the sum of one linear and one nonlinear function (Fig. 2).

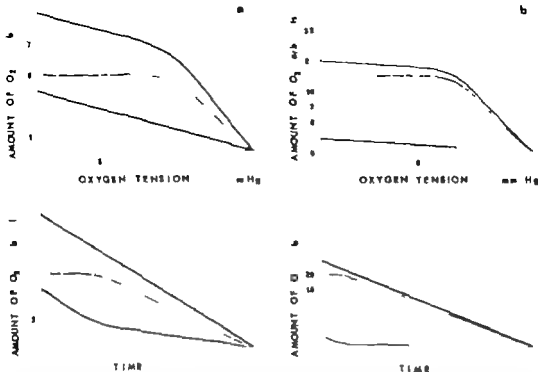


Fig. 2. In this figure the graphs are schematically drawn for an arbitrarily chosen hemoglobin concentration as well as for a certain physicochemical environment. The amount of physically dissolved oxygen, of hemoglobin-bound oxygen and the sum of these two fractions in a closed system related to oxygen tension (upper diagram in (a) and (b)) or time (lower diagram in (a) and (b)). In (c) the hemoglobin concentration is four times of that in (a). The upper solid curves in all diagrams represent the total amount of oxygen, the lower solid ones represent the physically dissolved pool and the dotted curves depict the hemoglobin-bound fraction. Note the nonlinear transformation of the graphs on substituting the independent variable time for tension and the increased proportion of the hemoglobin-bound oxygen at increased hemoglobin concentration. It can be seen that the change in shape of the dotted curves between upper and lower diagrams is less pronounced in (a), i.e. at low hemoglobin concentration. At very low hemoglobin concentration there will be virtually no change in shape of the curve.

Assuming constantly respiring material within the closed chamber the total amount of oxygen will decrease linearly when plotted against time. The function of the total O_2 -content has thus been nonlinearly transformed when substituting the independent variable time for O_2 tension. Since this transformation does not affect the proportions of the subfunctions (physically dissolved and hemoglobin bound O_2) at any arbitrary tension or time these functions can easily be reconstructed with time as the independent variable (Fig. 2). When the hemoglobin concentration is high the function for the relationship between time and the hemoglobin bound oxygen called $f(t)$ will be linear (Fig. 3) i.e.

$$\ln f(t) = k \cdot t \\ [Hb] \rightarrow \infty$$

On the other hand when the concentration approaches zero the function $f(t)$ will be

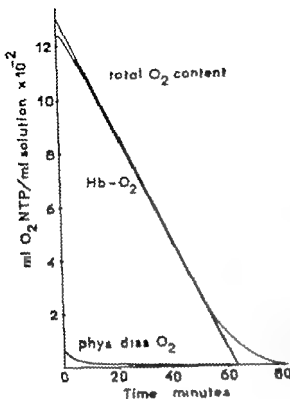


Fig. 3 Data from an actual experiment on the respiration of yeast cells in a microrespiration chamber the hemoglobin concentration being $1.36 \times 10^{-3} M$. It is seen that the absorbance shift curve is almost linear with time and that the physically dissolved fraction can be disregarded. The asymptotic approach to the absorbance is due to final cellular hypoxia. NTP: normal temperature pressure (from Hultborn 1972)

Identical to the function called $f(p)$ describing the relationship between the O_2 tension and the hemoglobin bound oxygen

$$\lim_{[Hb] \rightarrow 0} f(t) = f(p)$$

The graph describing the relationship between hemoglobin bound O_2 (i.e. O_2 -saturation) and time is the one recorded spectrophotometrically during the experiment

Calculation of O_2 -consumption from an absorbance shift curve (OD-curve).

A detailed description of the treatment of the spectrophotometrically obtained OD-curve based on the considerations above will be given in this section. In Fig. 4 the same graph as in Fig. 2 is presented: the dotted curve corresponding to an experimentally achieved curve and in Fig. 5 an actual recording is illustrated for comparison. The initial part is nonlinear due to the sigmoid shape of the dissociation curve and the final part has an asymptotic approach to the "reduced level" partly due to cellular hypoxia. For calculations the linear steepest part of the OD-curve corresponding to the region of half saturation is used. In this region the slope of the curve $\frac{\Delta OD}{\Delta t}$ is determined. Thus, during the time Δt a change in optical density ΔOD occurs corresponding to a certain consumed volume of hemoglobin bound oxygen, a volume which is calculated in the following way:

Knowing the oxygen binding capacity of hemoglobin, the hemoglobin concn

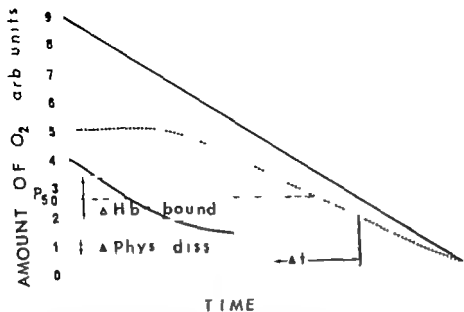


Fig. 4 Identical diagram to Fig. 2 () lower part. The amounts of the partial fractions of oxygen consumed per unit time in the system in the vicinity of half-saturation (P_{50}) are illustrated. The dotted curve corresponds to an experimentally obtained recording (OD-curve).

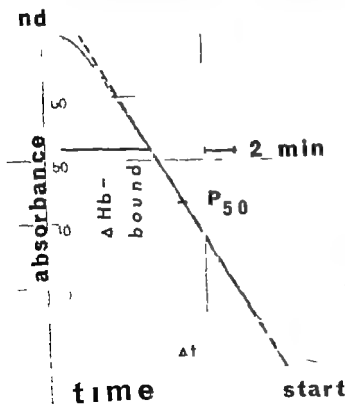


Fig. 5 An actual recording, OD-curve, from an experiment on isolated nerve cells. The fraction of the hemoglobin-bound O_2 pool as related to time in the vicinity of half-saturation is depicted. (Modified from Hultborn and Hydn 1974).

and the chamber volume the amount of hemoglobin bound oxygen (A) at complete saturation is known,

$$A = V \times [\text{HbO}_2] \times 68,000 \times 1.39$$

where V = chamber volume

$[\text{HbO}_2]$ = hemoglobin concentration (M)

68,000 = nominal molecular weight of hemoglobin

1.39 = oxygen binding capacity of hemoglobin (ml O₂/g Hb)

This volume of oxygen also corresponds to the hemoglobin bound oxygen consumed by the cells at complete deoxygenation of the hemoglobin. The optical density of the incubate will change to a certain extent from full oxygenation to complete deoxygenation. This change is denoted $\Delta \text{OD}_{\text{total}}$ and it corresponds to the consumed volume of oxygen called A. Thus the volume of oxygen corresponding to ΔOD is $\frac{\Delta \text{OD}}{\Delta \text{OD}_{\text{total}}} \times A$.

Since this volume of oxygen is consumed during the time Δt , the rate of consumption of hemoglobin bound O₂ is obtained.

When using moderate ($<10^{-3}$ M) or low concentrations of hemoglobin the physically dissolved fraction consumed during the time Δt must be added. This is most easily done by determining beforehand in calibration experiments, the slope of the dissociation curve $\frac{\Delta z \text{HbO}_2}{\Delta P \text{O}_2}$ ($z \text{HbO}_2$ denotes saturation of the hemoglobin which is directly

proportional to OD) by means of tonometry in the region of the P_{50} value. The reciprocal of the expression for this slope is proportional to $\frac{\Delta P \text{O}_2}{\Delta \text{OD}}$. From $\frac{\Delta P \text{O}_2}{\Delta \text{OD}} \times \frac{\Delta \text{OD}}{\Delta t}$ the expression $\frac{\Delta P \text{O}_2}{\Delta t}$ is thus achieved and the amount of physically dissolved oxygen that shall be added to the hemoglobin bound fraction is

$$\frac{\Delta P \text{O}_2}{\Delta t} \propto \frac{V}{760}$$

where α = Bunsen's coefficient for gas solubility of the medium (vol dissolved gas/vol. medium at a partial pressure of 760 mm Hg)

It is thus noted that no error is introduced when the hemoglobin concentration is low and thus the proportion of physically dissolved oxygen is increased since this fraction will be included in the calculations.

A correction of the volume of the incubation medium (V) might be necessary if large samples, displacing a significant fraction of the incubation medium are used.

Another approach of theoretical interest in the treatment of the OD-curve is the following. At the start of the experiment (zero time) the solution is equilibrated with air and the partial pressure of oxygen equals the volume per cent O₂ in air times the barometric pressure corrected for water vapour pressure. Since the hemoglobin is saturated at this pressure the oxygen bound to hemoglobin is known at zero time. Further from the solubility coefficient of oxygen the amount of physically dissolved oxygen at zero time is also known. Therefore and from the above considerations and Fig. 2 it is obvious that, whatever shape the oxygen dissociation curve has in the individual experiment it does not play any role with regard to the accuracy of calculations. Employment of these calculations also permits the determination of the oxyhemoglobin dissociation curves see p. 41.

Hemoglobin solutions

Hemoglobin is preferentially prepared in the laboratory since commercially available preparations are frequently highly contaminated by methemoglobin and may contain other impurities harmful to cellular metabolism.

Preparation and storage.

Various types of preparatory procedures to obtain hemoglobin solutions exist and the one adopted in this work is not claimed to be the most accurate one although it has proved to be adequate for the purposes of the techniques described in this work.

Human whole blood, stored for two weeks at 4°C with acid-citrate-dextrose (ACD)+adenine was used. Initial volume for each batch was 300 ml. The cells were rinsed with 0.15 M NaCl and centrifuged three times in the cold (1200 g for 10 min) whereupon they were rinsed with 0.17 M NaCl to decrease the cell volume. The cells were lysed by adding half the packed cell volume of redistilled water lysis being enhanced by shaking and freeze thawing. Cell debris were removed as a pellet after centrifugation at 50,000 g for one hour. The clear hemolysate (~50 ml) was then placed on a Sephadex G-25 column (Ø 2.5 cm, height 40 cm) equilibrated with the buffer solution intended for use in the biological incubation experiments or with 0.15 M NaCl these solutions also being used to elute the column. The effluent was collected in fractions of 10 ml which were then adjusted to pH 7.4. Taking the molecular weight of hemoglobin as 68 000 the maximal hemoglobin concentration of these fractions was between 2 and 3×10^{-3} M. The solutions were divided in 100 µl aliquots dispensed in small test tubes which are kept frozen at -80°C until used. At use the hemoglobin solution is diluted to the desired concentration in the incubation medium, often at relative volumes of 1:20 to 1:50 resulting in final hemoglobin concentrations in the range of 10^{-4} M.

Characterization.

The sodium, potassium and chloride content of the hemoglobin solutions should be analysed to check if the potassium rich hemolysate was adequately changed in ionic composition on the passage through the Sephadex column.

Concentrations of the hemoglobin solutions were determined by the two-wavelength method. Since a gradual autoxidation to methemoglobin occurs on storage the amount of oxyhemoglobin was determined after aeration of the sample. Absorption spectra from 600 to 400 nm were also recorded in oxygenized and reduced state for comparison with data in the literature. In the study presented here a Zeiss double beam spectrophotometer was used for this purpose and extinction coefficients for hemoglobin and its derivatives were obtained from Assendelft (1970).

Crude hemoglobin-oxygen dissociation characteristics were also determined spectrophotometrically by use of a simple tonometric procedure. The hemoglobin solution in the incubation medium, (~3 ml, concentration and composition as for an actual biological

experiment) is gassed with pure nitrogen for 20 min after which a spectrum from 600 to 500 nm is recorded (similar to that of a sample reduced by sodium dithionite). After subsequent bubbling, each 5 min, with gas mixtures (1.10 % O₂ & 98.90 % N₂, 1.89 % O₂ & 98.11 % N₂, 5.46 % O₂ & 94.54 % N₂ and air) the spectra of the solution from 600 to 500 nm are recorded. From the data obtained an estimation of the half-saturation value of the hemoglobin can be derived as well as the steepness of the curve $\frac{\Delta x \text{ HbO}_2}{\Delta P \text{ O}_2}$ essential for the calculation of the oxygen consumption as discussed in the previous section. Typically P₅₀ values obtained in these series of investigations were between 10 and 15 mm Hg and $\frac{\Delta x \text{ HbO}_2}{\Delta P \text{ O}_2}$ values were about 0.04 mm Hg⁻¹. These dissociation characteristics should be determined whenever experimental conditions are changed (e.g. hemoglobin concentration, media constituents pH etc.)

Determination of O_2 -consumption in the range of $1 \mu l O_2/h$

This section deals with the principles and procedures in determination of respiratory rates of cell suspensions and tissue homogenates, i.e. of particulate suspensions which will present specific problems when compared to the measurements of O_2 -consumption of well-defined solid tissue samples treated in later sections.

Work with particulate suspensions introduces two major spectrophotometric artifacts, firstly there occurs a decrease of transmission of the incident light due to the presence of particles which diffract and absorb part of the light (c.f. Fog 1952 Wodick and Lubbers 1973). Since biological suspensions are extremely complicated in composition and structure no mathematical expressions for the degree of transmission can be used. Furthermore a change in diffraction and absorbing properties with time might be present. These complications are circumvented in the present system by the fact that no absolute absorbance values are required calculations are made purely on *changes* in absorbance of the same incubate (ΔOD). Furthermore a time-dependent change in diffraction is compensated for by use of a double beam spectrophotometer where the same biological suspension without indicator pigment is placed in the reference beam. The second spectrophotometric as well as respirometric artifact is due to particle sedimentation in the incubation chamber (cuvet). In conventional spectrophotometers this leads to a continuous passage of particles across the measuring light beam and to an increasing or decreasing concentration of particles in the volume traversed by the light beam. This can to a great extent be compensated for by a simple rearrangement of the measuring beam to a vertical direction so that the light passes through the cuvet in the same direction as the particles settle. Further using short sedimentation distances the time of particle movements can be greatly decreased. The respirometric error introduced by particle sedimentation is due to aggregation and increased diffusion distances. In the type of cuvetas described below this fact will not be of importance since almost a monolayer of particles will be present when sedimentation is finished. The measuring system described in this section is also equipped with an automatic cuvet changing device to increase measuring capacity.

Incubation chambers (cuvets)

The incubation chambers used consist of commercially available fused 1 mm pathlength optical cuvetas (visible light) with the outer dimensions $12 \times 45 \times 3$ mm. Suitable working volume of these cuvetas is 200–300 μl . The cuvet is sealed by the introduction of approximately 50 μl of liquid paraffin. The liquid in the cuvet is held by capillary force so that the cuvetas can be placed horizontally without risk of leakage. After the experiment the cuvetas should be cleaned as soon as possible. The contents are thrown out by hand and the cuvetas are then rinsed in running hot water for half a minute. The cuvetas are then immersed in a detergent solution for one day after which they are repeatedly rinsed in tap water, reimmersed in absolute ethanol for at least one day and finally rinsed several times in distilled water and dried. Immersion of the cuvetas is most suitably carried out in individual plastic vessels to avoid damage to the optical surfaces.

Analysing equipment

Basic unit A double beam recording spectrophotometer (Zeiss DMR 71) is used as the basic equipment. Monochromatic light is produced by a prism monochromator (M 4 QIII) with an automatically regulated slit width to give a constant reference signal which in turn can be altered by changing the degree of amplification. For the special purpose of the technique described, the whole interior of the cell compartment was removed and replaced by components described below.

Modifications. The alterations and new constructions mainly concern the cell compartment thus the rest of the instrument is only briefly interfered with. The aim of the modifications was to combine the following four demands:

- a) to measure on horizontally positioned cuvetts of the type described above
- b) to produce a versatile piece of equipment for use with conventional cuvetts
- c) to have an automatic cuvet changer accommodating up to five samples and five references functioning both for horizontally positioned cuvetts and conventional ones
- d) to control temperature in both types of cuvetts

The automatic cell changer is provided with two pairs of cuvet-wheels, one of the pairs with holders for 1 mm pathlength cuvetts in horizontal position (*i.e.* for respirometry (Figs. 6-7-8)) and the other with holders for conventional cuvetts. Both wheel-pairs are placed on a plain brass plate to which a synchronous motor is attached. The brass plate is fastened to the bottom of the cell compartment. Each cuvet-wheel has positions for five cuvetts and each one can be in a measuring position from 5 to 60 sec. varied by means of a timer within the control unit (Fig. 9). The bottom brass plate is provided with a zig-zag channel for circulation of temperature regulated water.

In the set-up for respirometry the light-beams passing through the cuvetts have been changed to a vertical direction by an optical system including four plane surface coated mirrors in each beam. Two mirror-holders are mounted on the brass plate above the cuvet wheels by means of long knurled screws. Each holder is provided with two mirrors inclined 45° to the horizontal plane, the positions corresponding to two similar mirrors mounted in a groove in the brass plate. Each light-beam is thus reflected four times within the cell compartment and the cuvet is placed above a circular hole (10 mm \varnothing) in the wheel between the third and the fourth mirror. A stationary aperture (8 mm \varnothing) determines the dimension of the light beam in the plane of the cuvet while another diaphragm in front of the mirror holder prevents any direct light from passing to the light detector.

To allow for an automatic cuvet changing function (Fig. 6) shaft pivots of the sample and reference wheels (gear wheel 100 cogs, 100 mm \varnothing) are placed on the bottom-plate so that the wheels gear while one of them is driven by another gear wheel (40 cogs) connected to a synchronous motor (RSM 50/8 NG Berger Lahr West Germany) which has a speed of 5 r.p.m. (cuvet wheels 2 r.p.m.). To make the wheels stop at appropriate positions there is a sensing lever fitting in excavations in the lower surface of the sample wheel corresponding to the positions of the circular holes in the wheel thereby influencing a microswitch which disconnects the motor via the control unit (Fig. 9). When in correct position recording is performed after a delay of 3 sec. of a timer. To measuring system to stabilize. Time of measurement can be set by use of a timer. To avoid unnecessary wear of the potentiometer and monochromator slit motor these units

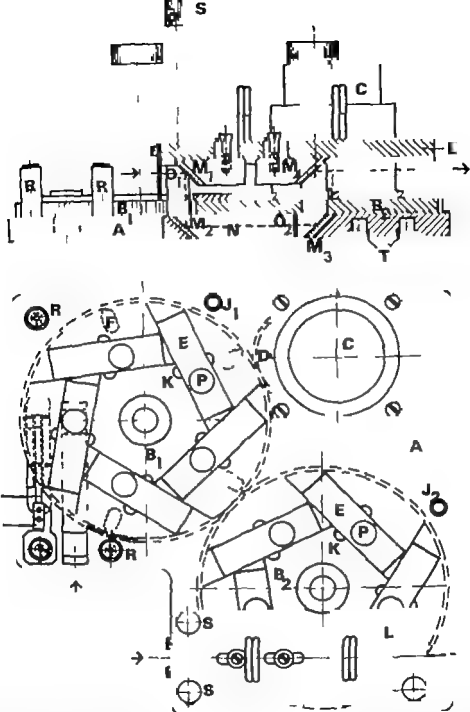


Fig. 6. Cuvet-changer arranged for spectrophotometry with horizontally positioned cells, lateral and vertical projections. A beam-plate, B and B₂ sample and reference cuvet-wheels, C synchronous motor, D driving gear wheel, E cuvet in position, F excitation lever, G sensing lever, H microswitch, J₁ and J₂ inlet and outlet for air, I and I₂ spaces for tweezer-grips, L mirror holder, M₁-M₄ mirrors, N groove for light beam, O₁ and O₂ apertures, P holes in cuvet wheels for light passage, R, pillars for mirror holders, S knurled screws, T channel for temperature-regulated water. (Modified from Hultborn *et al.* 1974)

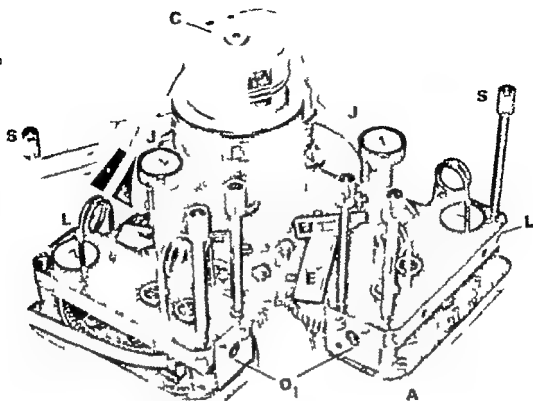


Fig. 7 The cuvet-change assembly completely assembled for spectrophotometry. Symbol as in Fig. 6 (from Hultborn *et al.* 1974).

are disconnected during cell-change. The control unit operates at low voltage. To allow an appropriate temperature regulation of the cuvetts a channel (5×5 mm) is milled in the brass plate for circulation of water (Fig. 8). The heated brass plate transmits the heat to the cuvet wheels and the cuvetts via the large contact area between the wheels and the brass plate.

Working characteristics. The accuracy of cuvet repositioning, i.e. cuvet wheel stopping, was examined for each cuvet position in the sample and reference wheel. The angle accuracy is $\pm 0.06^\circ$ (1 SD) which is not critical especially since the light-beams will pass perpendicularly to the cuvet in any case and since the apertures are so arranged that the holes for the light beam in the cuvet wheels are not limiting.

The extended pathway of the light beams (due to the mirror system), the reflections and the introduction of apertures decrease the amount of light passing through the cell compartment. When there is no diaphragm in the beam the reduction of energy was found to be equivalent to that obtained when conventional microcells are used. Since the instrument has a sufficient stability the decrease in energy does not cause any inconvenience in the type of measurements performed.

The efficiency of the thermostatically controlled system was tested at an ambient room

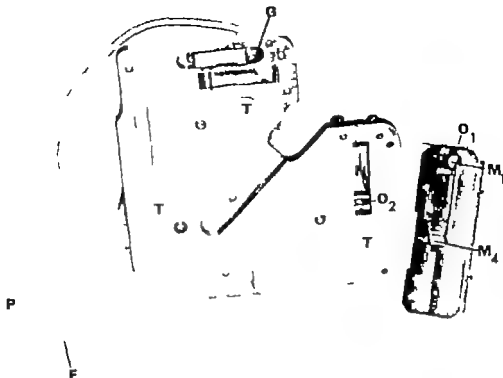


Fig. 8 From left to right the lower surfaces of sample cuvet-wheel for spectrophotorespirometry the brass bottom-plate and one mirror holder. Symbols as in Fig. 6 (From Hultborn *et al.* 1974)

temperature of 20°C and the water was circulated at 37°C at a rate of 1.3 l/min. After one hour of equilibration without using the lid of the cell compartment there was a difference between inlet and outlet water of 0.1–0.2°C. No difference in temperature (<0.1°C) between sample and reference cuvetts was found as studied with thermistors as well as encapsulated liquid crystals. The cuvetts attained a temperature less than 0.5°C below that of the inlet water.

Procedure

To a certain volume of the suspension which is to be analysed concerning its oxygen consumption is added a much smaller known volume of the concentrated hemoglobin solution. The final hemoglobin concentration is thus known. To ensure homogeneous distribution of biological material and pigment in the suspension as well as to complete the saturation of the hemoglobin with oxygen the solution is shaken and 200 to 300 µl of the suspension is transferred to the sample cuvetts by means of a Pasteur pipette. Liquid paraffin is placed on top of the suspension. The cuvetts should immediately be placed in horizontal position to allow sedimentation of particles and if a rapid respiratory rate is expected, it is advisable to place the cuvetts on a cooled surface. The

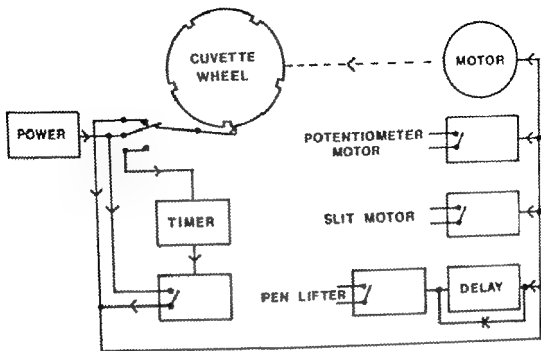


Fig. 9 Block diagram of the electro-mechanical units of the cuvet-charger (From Hultborn *et al* 1974).

reference cuvettes are filled with the same biological suspension as the sample cuvettes, but do not contain hemoglobin and subsequently sealed as above. The cuvettes are then gently placed in their correct positions on the cuvet-wheels by use of a pair of tweezers. A spectrum from 600 nm to 500 nm is recorded to identify the oxyhemoglobin, whereupon the recording of the absorbance at 435 nm commences and continues until the absorbance change is finished. Another recording from 600 nm to 500 nm is performed to ensure that complete deoxygenation of the sample has occurred.

Differential spectrophotometry

Use of a double beam spectrophotometer permits the recording of a difference spectrum (Hartmann 1937) of two "active cuvettes" *i.e.* both sample and reference cuvettes contain biological material incubated in the same concentration of hemoglobin. This procedure enables the identification of differences in respiratory rates of two suspensions which are identical except for the reactant or inhibitor the effect of which is to be studied. Initially the contents of the two cuvettes are oxygenated, so that the instrument indicates zero absorbance at any wavelength. Thus any deflection in absorbance from zero measured at any wavelength except the isosbestic points indicates a difference of respiratory rates of the two suspensions (Fig. 10). Recording at a constant wavelength preferably 435 nm will in case of different respiratory rates result in a bell-shaped curve returning essentially to the initial absorbance value (zero), when the hemoglobin is completely reduced in both cuvettes. In the sample (experimental) as well as in the

absorbance

1

2

600 500 400
wave length nm



Fig. 10 A repeated wavelength-scanning differential-spectrophotometry of leucocytes incubated together with endotoxin in the sample cuvet to induce respiratory burst. Upper curves show the reaction of leucocytes from a normal individual, while the lower curve illustrates the absence of increased respiration of leucocytes from individuals suffering from chronic granulomatous disease. A wavelength scanning has been performed every 7th minut from 600 nm (left) to 400 nm (right). The difference between the reactions of normal and abnormal individuals can, with greatest accuracy be seen at 435 nm. (From Hultborn and Olling 1973).

reference (control) cuvet there will be an increase in absorbance with time. The total increase in absorbance from full oxygenation to complete deoxygenation is equal for both cuvet and is known from the hemoglobin concentration which also characterizes the shape of the absorbance shift curves, as described on p. 10. The bell-shaped curve is, in fact, the result of a subtraction of the absorbance shift curves of the two cuvet, and since the hemoglobin concentration is known they can roughly be reconstructed. However, the amplitude of the deflection from the base line (zero) can be used as an indicator of the difference in the rates of hemoglobin reduction in the two cuvet. It must be emphasized that it is important to test that the substance whose effect on the respiratory rate is to be studied does not itself influence the oxygen affinity of the hemoglobin since this introduces erratic results for two reasons. Firstly, the actual measurement of respiration would not occur at the same O_2 tension in the two cuvet and this might change the respiratory rate. Secondly, even if the respiratory rates were the same, the desaturation of the hemoglobin and thus the increase in absorbance would start earlier in the cuvet with the lowest O_2 affinity of the hemoglobin which would give rise to a deflection from the base-line.

Accuracy

An analysis of the slopes of the steepest parts of the absorbance shift curves (OD-curves) from ten experiments with a common yeast cell suspension yielded a remarkably good reproducibility $\pm 2.2\%$ (1 SD) for the instrumental system together with the dissociation kinetics of the hemoglobin and pipetting errors (Hultborn *et al* 1974).

The differential-spectrophoto-respirometry should as yet be regarded as a semiquantitative yet sensitive method for detecting differences in respiratory activity of suspensions of common biological material

Final remarks

The technique described in this section was developed using a Zeiss double beam spectrophotometer but it should be emphasized that a similar set-up can be made with other types of instruments, even single beam spectrophotometers can be used for the direct quantitative measurements. Another possibility would be to use the described cuvet changer as a separate unit connected to any spectrophotometer by the use of fiber optics. In this technique an instrument is utilized which is not exclusive for respirometry like most manometric equipments would be but can also be used in other spectrophotometric work. The method is easy to handle and not too sensitive to external influences, thereby making it suitable for routine use without special training

Determination of O_2 -consumption between 10^{-2} and 10^{-6} $\mu l O_2/h$

The technique described in this section is applicable to well-defined small tissue samples down to isolated single cells in contrast to the technique described in the previous section. The measuring light-beam will in these cases not pass through a turbid solution, and the majority of spectrophotometric problems discussed above are thus not present.

The main outlines of the technique concern incubation of the tissue samples in minute chambers and the recording of the absorbance change of the hemoglobin solution in the chambers by means of a single-beam microscope spectrophotometer

Incubation chambers (cuvets).

General requirements

- The volumes of the incubation chambers must be between $1 \mu l$ and $10^{-4} \mu l$ and be well defined
- It must be possible to close the chamber so that no leakage of gas occurs.
- The material in the walls of the chamber must be inert to oxygen so that no gas diffuses into the chamber from the walls. This is more imperative the smaller the incubation volumes are since the proportion of wall area to chamber volume is inverse to the volume
- At least two opposite facets of the chamber must be transparent to visible light to allow the measuring beam to pass through

The depth of the chamber i.e. the optical pathlength of the chamber should not be small in relation to other dimensions of the chamber (e.g. in the case of cylindrical chambers the length of the cylinder should be in the range of or larger than the diameter). The reason for this requirement is twofold firstly to obtain a sufficient degree of absorbance without increasing the concentration of hemoglobin dramatically the optical pathlength should be as long as possible without increasing the volume of the chamber secondly the loading and closure of the chamber discussed later will be easier with the above mentioned geometrics.

- The chambers should not be too delicate since they must be handled and cleaned after each experiment

It proves difficult to meet all these requirements adequately though the solutions described below fairly well fulfill the demands. As for the material of which the chambers are produced, it turns out that glass and synthetic corundum, (crystallized Al_2O_3) are sufficiently inert to oxygen which is not the case for any plastic material. The surfaces of glass and corundum can furthermore be polished so that appropriate closure of the chamber is obtainable and the material is transparent to visible light.

Chambers used for O_2 -uptake in the range of $10^{-2} \mu l/h$. A suitable chamber volume for work in this range is approximately $1 \mu l$. One such type of $1 \mu l$ chamber can easily be produced by use of sintered carbide drills having a diameter of approximately 1 mm . A bore is produced through a plane-parallel approximately 1 mm thick glass slide (conventional object slide) using a precision drilling machine. To avoid cracks in the glass

surface the slide must be drilled from both sides, and to ensure that the drilled holes will meet perfectly a small excavation is first drilled in a metal-plate fixed in the stage of the drilling machine. The glass slide is placed on this metal-plate and a bore is made to approximately half the thickness of it. The slide is removed and a small sphere with a diameter similar to the drill is placed in the excavation of the metal plate. The glass slide is turned upside down and placed so that the bore fits with the sphere during drilling. Slides with bores having cracks are discarded since volume determination of them would be difficult. The volume of the bore (chamber) is achieved from mechanical micrometer determination of the thickness of the slide and microscopic determination of the bore diameter. To produce a bottom to the chamber a cover slip is fastened on to one side of the object slide with Nobocutan® (Bofors Nobel Pharma, Mölndal Sweden) sucked in between the two glasses by capillary force. As a cover of the chamber an object slide of a smaller size is firmly pressed on to the other glass. To be sure that the cover is tightly fitting, it is checked that interference fringes readily occur when juxtaposing the two object slides.

The described production of drilled chambers is suitable for multiple bore drilling in each glass-slide for use with an automatic cuvet-changing device described below. The bores should then be placed in line with regular distances between each other conforming to the steplength of the cuvet changer. Slides with six bores with distances of 3 mm have been used in this laboratory.

Another type of chamber suitable for this range of work is made of synthetic corundum the sapphires being purchased of Seitz SA, Les Brenets, Switzerland. The "hole jewels" used for this range of measurements have bore diameter of 1.00 mm, outer diameter of 3.20 mm and thickness (i.e. height of the chamber) of 0.80 mm though other dimensions can be obtained as well as is indicated below. The upper and lower surfaces of the sapphire are polished so that adhesion to an optically plane surface can be achieved by rubbing. The surfaces have also been controlled by interference microscopy. Thus no adhesive is needed to produce a bottom. The jewel can simply be placed on an optically plane glass slide (cover glasses for hemacytometers, 0.6 mm thick, 10 × 20 mm) and charged with the sample. As cover a smaller sized 5 × 5 mm glass slide of the same type is used.

Chambers used for O_2 -uptake in the range 10^{-4} – 10^{-6} μ l/h. Chambers used for this range of respiratory rates suitably have volumes between 10^{-3} and 10^{-6} μ l and due to these small volumes they can hardly be produced in the workshop of an ordinary laboratory. Synthetic sapphire jewels with a precision bore in the centre (as above) can, however, be purchased in various dimensions (Seitz SA, Les Brenets, Switzerland). Two dimensions of sapphire chambers have been used in this study having internal diameters of 0.250 and 0.040 mm, external diameters of 1.00 and 0.90 mm and thicknesses of 0.250 and 0.150 mm respectively. The larger sized chamber having a volume of 1.3×10^{-3} μ l (Fig. 11) is suited for measurements of respiratory rates in the range of 10^{-4} μ l/h, while the smaller one is suited for the range of 10^{-6} μ l/h. The surface finish of these chambers is the same as for those described in the paragraph above. Since the sapphires are birefringent they can easily be visualized with polarized light which in the case of uncoloured jewels may be of importance (see below). The bottom and the cover of these sapphire chambers are arranged in a similar way to that described in the previous paragraph.



11. A sapphire jewel chamber with internal diameter and depth of $250\text{ }\mu\text{m}$ used for measurement of the respiratory rate of isolated Deltans nerve cells. The cell, its nucleus and nucleolus clearly visible within the chamber

In collaboration with Carl Zeiss (Oberkochen, West Germany) another type of "ultramicrochamber" has been produced in quartz glass. A thin ($70\text{ }\mu\text{m}$) bored quartz glass is fused to another optically plane quartz slide. The bore diameters vary from 60 to $100\text{ }\mu\text{m}$, and the outer diameter of the glass is approximately 4 mm . The prime difference between the fused chambers and the sapphire chambers described above is that in the former type the chamber consists of one single unit. In contrast to the separate sapphire top and bottom in the latter type.

The outer dimensions of the fused type of chamber are larger which make them easier to handle but the cleaning procedure for the sapphires is easier since they are both mechanically and chemically highly resistant. By means of Pasteur pipettes (tip diameter slightly greater than that of the sapphire) they are sucked up and immersed in NaOH 0.2 M for a few hours, in HCl 0.2 M for one day, in ethanol and finally in distilled water for two days. The fused chambers are more fragile and care should be taken not to expose them to alkaline solution or ultrasonic procedures. These chambers are more difficult to clean since they constitute a blind bore in contrast to the penetrating bore of the sapphires.

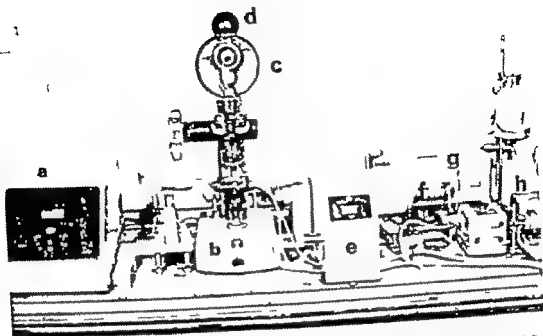


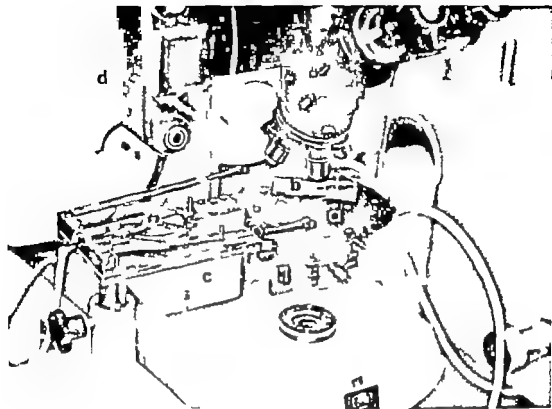
Fig. 1. Complete set of the microspectrophotometric equipment used. From left to right: amplifier (a), microscope (b) with photometer head (c) and photomultiplier (d), monochromator (e), light source (f) with voltage stabilizer (g) and recorder (h).

Due to the restricted experience concerning the use of the Zeiss quartz-chambers they will not be further discussed in this work.

General detecting equipment

A survey of the various components of the basic equipment used for microspectrophotometry in this type of work will be presented below. It should be emphasized that most parts of the system are commercially available and that the whole equipment can be used for other purposes than oxygen consumption measurements, such as ordinary microscopy, microphotography, cytophotometry, etc. without any alterations of the arrangements (Sorby 1867, Loofbrouwer 1950, Freed 1969, Pollister *et al.* 1969). Photographs of the instrumentation are shown in Fig. 1-3. In this equipment Zeiss details have been compiled together with some components manufactured at the workshop in the Laboratory.

Light source. Since the technique utilizes the visible range of electromagnetic radiation to which photomultiplier tubes are extremely sensitive and since the measured substances are fairly large, the light source need not be of special high intensity. Of great importance in work with single beam instruments in studies of time dependent reactions is to achieve a light source providing constant emission. Arc lamps of various types are not suitable as the intensity and position of the arc frequently changes and thus the zero and 1



3 Close-up view of the equipment. Temperature regulated object stage (a) with clamping tool (b) the left the cuvet-changing device (c) and camera (d) fastened on to an extra side-viewing box.

transmission settings can not be relied upon. Filament (incandescent) lamps are therefore preferred, but the use of low-voltage sources makes it necessary to ensure that connections with the voltage supply are adequate (preferably soldered). Voltage stabilizers should be used in all cases. An auxiliary polychromatic illumination should also be provided for the purpose of centering and focussing an area of the chamber not occupied by the sample.

Monochromator Various devices such as filters, grating or prism monochromators can be used for spectral isolation. In the present situation, where analysis of absorbance differences of hemoglobin compounds having rather sharp absorption characteristics, is to be performed, it is advisable to use good instruments providing facilities for changing band widths and exact setting of wavelength. Interference filters are therefore less suitable for this type of work. By increasing the band width less amplification of the cathode-current of the detecting photomultiplier is needed. The diminished spectral resolution does, however, result in a decrease in amplitude of the absorbance shift curve. Furthermore, it is of importance to have the visual field (object plane) uniformly illuminated with respect to wavelength. This can be done by means of a prism- or grating monochromator with the exit slit as light source, i.e. according to Kohler illuminated with respect to wavelength. This can be done by means of a prism

Most commercial grating or prism monochromators fully meet the above mentioned demands. In the present study a Zeiss prism monochromator (M 4 QIII) was used.

Microscope and optics. Any mechanically stable microscope with facilities for flexible Kohler illumination can be used. Side viewing tubes, one for ocular inspection of the specimen, should also be provided. Above the object stage there must be ample space to mount a temperature regulated plate. Centering and focussing facilities of the condenser (most suitably a low power objective) must be provided as well as the possibility of inserting various field iris diaphragms to obtain suitable dimensions of the measuring light-beam. Since measurements are made in visible light conventional optics can be used. Measured areas are always fairly large ($>500 \mu\text{m}^2$) and thus low-power objectives are used. The condenser should have a low numerical aperture since an increase in actual optical pathlength in relation to the nominal one otherwise will occur (Blout 1950). Achromatic lenses are fully sufficient for recording, though apochromatic ones are convenient when characterizing the spectrum of an incubate.

In this study a Zeiss UVM II microscope was used with a $10\times$ N.A. 0.2 objective as condenser and as objective an apochromate $6.3\times$ N.A. 0.16. Field inses varied from 0.1 mm to 0.3 mm in diameter resulting in diameters of $15 \mu\text{m}$ to $45 \mu\text{m}$ in the object plane.

Photometer head and detector with amplifier. The photometer head should provide facilities to inspect the image of the area to be measured. Furthermore there should be different apertures, so that the area measured can be varied. Electron multiplier tubes are excellent as detectors for measurements in visible light since they have a very high quantum efficiency. The electronics should provide the possibility of varying the overall voltage of the photomultiplier tube as well as of changing the amplification of the anode-current. It is preferable to have the light modulated (e.g. by a beam-chopper) which allows the signals to be amplified using sharply tuned amplifiers with consequent rejection of much noise and diminished sensitivity to stray light. The instrument should also be equipped with a galvanometer. An RCA photomultiplier tube 1 P 28 was used in this work, generally at an overall voltage of approximately 580 V. The amplifier light modulator and photometer head was of Zeiss MPM type.

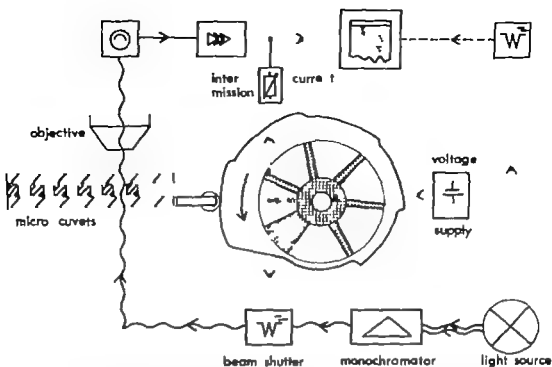
Recorder. Since the absorbance change of the preparation studied is proportional to the relative amounts of the two hemoglobin compounds it is convenient to have a logarithmic transformation (from transmission to absorbance units) of the transmission signal fed from the amplifier. Thus, the graphs obtained from the 3rd order can be calculated upon directly. In certain instruments this "transformation" may be made at an earlier stage by holding the anode-current of the photomultiplier constant in which case the dynode voltage becomes a logarithmic function of the input light intensity. The dynode voltage is then used as a measure of absorbance.

The recorder should furthermore have an electromagnetically controlled harp-pen lifter easily made by the workshop of the laboratory for use with an automatic rate changing device. A Vitatron UR 405 Linear Logarithmic recorder was used in this work.

Special equipment

Temperature regulation of the object. Since cellular O_2 -uptake is highly temperature dependent it is essential to be able to control incubation temperature.

detector amplifier recorder pen lifter



14 Block diagram of the opto-electro-mechanical unit in the microspectrophotoreaspirometric system including the automatic cuvet changing device. The program chart and cam are housed in the centre. The dotted areas on the program chart are copper laminated and serve to open the circuit for auxiliary functions during cuvet change.

limits. Various electrically heated object stages are available but most of them do not permit easy handling of the preparation. Thus, it has proved most suitable to use a metal panel with channels inside connected with cold tap water and with a thermostatically precision regulated circulation waterbath. Temperature is calibrated by the use of melting substances within the incubation chambers (e.g. Eicosan $\text{CH}_3(\text{CH}_2)_{18}\text{CH}_3$ melting point 36.8°C which must be exactly determined for each batch purchased). The melting substance is placed in the chamber under similar conditions as the biological samples and during continuous microscopic visual inspection of the crystals, the temperature of the circulation waterbath is slowly increased until disintegration of the crystals occurs.

Automatic cuvet changing equipment To increase the measuring capacity a cuvet changing system has been developed as yet only used in determinations of O_2 -uptake in the range of $10^{-2} \mu\text{l/h}$ (see p. 25). The system is based on a cam mechanism with a disk cam ground to precise dimensions (six different radii with 3 mm steps) and a radial roller follower directly connected to the object stage. The cam is driven by a synchronous motor at a speed of 0.5 r.p.m. resulting in a measurement of all six chambers every two minutes. A beam-shutter prevents the photocathode from exposure to too intense a radiation during displacement (cuvet changes). Simultaneously a variable intermission current is fed to the recorder to avoid infinite absorbance. Slightly before the

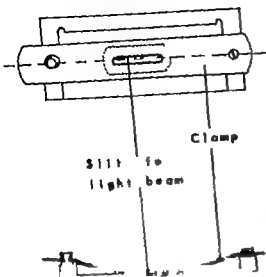


Fig. 15 The clamping tool used to press the cover glass on to the object slide with drilled chamber(s) as seen from above and the side.

beam-shutter and intermission current are operated the chart pen is raised electromagnetically. The electronic functions are controlled mechanically by sliding contacts, following the rotating cam. For details see Fig. 14.

Procedure

Loading of chambers drilled in object slides. Before introduction of the tissue sample a chilled chamber is filled with incubation medium including oxyhemoglobin. The solution is introduced into the cavity by means of a pipette the tip diameter approximately 0.5 mm (pulled manually in a flame) so that the tip reaches the bottom of the chamber. A rather large volume ($\sim 50 \mu\text{l}$) of the medium is blown out so that the chamber is filled, and a large surplus amount of solution is situated on the glass surface around the chamber. At this stage it should be checked that no air bubbles are trapped at the bottom of the chamber or on the walls, using phase contrast microscopy (low power objective). If a few bubbles are present they can easily be removed by means of a sharply pointed stainless steel needle. The tissue sample can then be introduced into the chamber under a stereomicroscope. The sample sediments rapidly to the bottom of the chamber and another check for air bubbles sticking to the sample should be made. A smaller sized object slide is then applied as cover of the chamber by means of a pair of tweezers, and the surplus hemoglobin solution is pressed away. If this moment is considered critical a tool described in the paragraph below may be used. Before the cover glass is firmly pressed on to the "chamber slide" it is once again checked that no air bubbles are present in the chamber. The firm pressure is produced by the tool depicted in Fig. 15. The elongated slits in the tool serve for use with multiple-drilled glasses together with the cuvet-changing device described above. The loading of the multiple-bore glasses is similar to that described above. Care should however be taken to have the samples in the row of bores, all situated in the "upper or lower regions of the chambers, so that the meaning beam will not be blocked by the sample in one or several chambers. This localization is achieved by tapping on the tilted compressed preparation which is

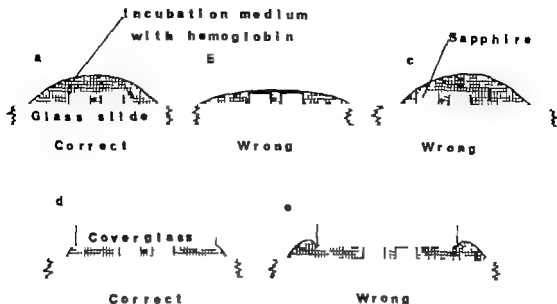


Fig. 16. Illustration of how the sapphire chamber should be located (a) in the drop of medium covering the whole sapphire (upper row). Too small amounts of medium (b) or an eccentric position of the sapphire in the drop (c) will cause disturbing turbulence of the liquid during the covering procedure resulting in escape of sample from the chamber. Too large a drop on the other hand will cause the cover glass to float on the surface of the liquid with sample loss as a consequence (e).

on the object stage of the microscope. When using the cuvet-automaton it is important to orientate the preparation so that the row of bores exactly parallels the line of back and forth movements of the object stage. Focussing and centering the specimen is performed using polychromatic light. The field iris aperture is introduced and monochromatic light of 435 nm is allowed to enter the system. The amplification is then adjusted so that the galvanometer indicates an arbitrarily chosen low degree of absorbance (the recorder should cover the range of increase of absorption without changing settings during recording). Recording is then commenced and continued until a new stable ("reduced") level of absorption is achieved. The absorption spectrum of the solution in the chamber should occasionally (especially when starting a new experimental series) be plotted both before and after the experiment. The amplitude of the absorbance shift curve obtained (OD-curve) should also conform to the theoretically calculated value derived from the hemoglobin concentration.

Loading of sapphire chambers. Since the sapphires are not fixed to the underlying plane-parallel glass slide the filling procedure of these chambers will differ from that above. The incubation medium will in this case cover the whole jewel so that this is immersed in the hemoglobin containing drop. For the large sapphire bores this is easily done with a pipette as in the above paragraph. For the smaller sized bores ($< 250 \mu\text{m}$ and $40 \mu\text{m}$) the chambers must be filled by means of delicate narrow tipped micropipettes. These are pulled in a classical electromagnetic puller intended for making glass microelectrodes. It is important to fill the bore first and not to immerse the whole jewel once since the trapped air in the bore will be difficult to remove. The micropipette is charged from above by a syringe and is operated either by mouth or by a syringe with a

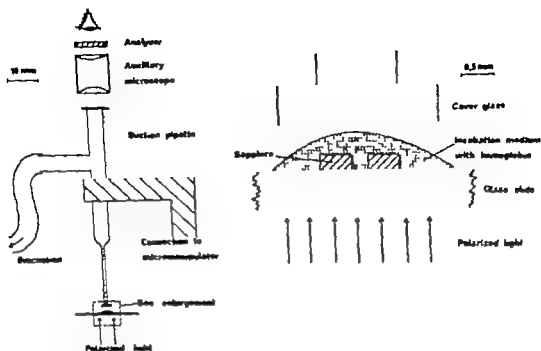


Fig. 17 Illustration of the equipment used to cover the sapphire chambers. The procedure minimizes liquid turbulence and consequently the risk of losing the biological material at this step. For the procedure see the text.

three-way stopcock. The filling is then carried out freehand under a stereomicroscope. The way how the immersed sapphire should be located within the medium drop as well as some erroneous positions are depicted in Fig. 16. Since small volumes of aqueous solutions are handled it is of importance to avoid excessive evaporation. Hence the relative humidity of the laboratory should exceed 60 per cent and use of humidity chambers (moist Petri dishes) between operative steps is advised. The sample can now be introduced into the chamber through the surplus solution surrounding the sapphire a procedure which generally has to be done under stereomicroscope. After spontaneous sedimentation of the sample to the bottom, which occurs rapidly the preparation is ready to be covered by a small sized glass slide. Since the sample specimens are very small and the sapphires are not fixed to the glass underneath it is of importance not to cause too much turbulence of the liquid, when applying the cover a situation which may cause the sample to be sucked out of the chamber or even displace the whole sapphire. A special tool was therefore constructed to minimize liquid turbulence. As seen in Fig. 17 the cover glass is fastened by suction to a pipette connected to a manipulator movable in three directions. By an auxiliary microscope it is possible to see through the pipette along its axis, so that the cover glass can be exactly centered above the sapphire. To visualize the small inconspicuous, birefringent sapphire it is illuminated from below by polarized light. The pipette is slowly lowered so that the incubation medium makes contact with the cover glass and is then further lowered until the cover glass touches the sapphire. The rubber tubing connected to the suction pipette is compressed manually and after a few seconds

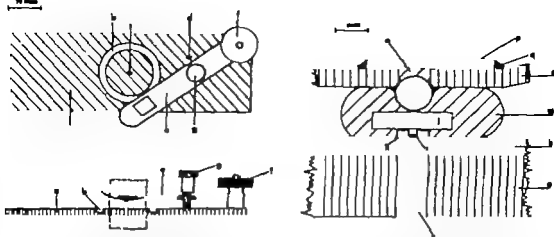


Fig. 18 Schematic drawing of the tool used to clamp the incubation chamber preparation. To the left top and lateral views of the whole tool are shown, and to the right an enlargement of the marked region is depicted. Plane-parallel brass plate (a) circular groove (b) central bore (c) excavation (d), clamp (e) bolt and knurled nut (f) knurled screw (g) plano-parallel glass slide (h) central bore in the sapphire, i.e. the incubation chamber (i) sapphire jewel (k) plano-parallel cover glass (l) immersion oil (m) glass sphere (n) adhesive or immersion oil with the same refraction index as the glass sphere (o) cover glass (p) adhesive (q). (From Hultborn and Hydén 1974).

the cover glass has detached from the pipette so that this can be raised. To avoid instability or even floating of the cover not too large a drop of incubation medium could be used (Fig. 16). The preparation is inspected under a microscope to confirm that sample has not disappeared and that no air bubble has been introduced. The cover of the chamber should be firmly pressed on to the sapphire to prevent gas exchange with the environment. This clamping is fulfilled by a tool specially designed to attain accurately centric pressure (Fig. 18). The principle of the tool is to let the measuring light-beam, passing the chamber also pass through a glass sphere fused in a hole in the clamp thus ensuring a central pressure. In spite of the relatively complicated optical system the image of the chamber is not much distorted, provided immersion oil is introduced in the system as indicated in Fig. 18. The whole preparation is now ready for transfer to the object stage of the measuring microscope. Further procedures are similar to those described in the previous paragraph.

Addition of reactants in the course of an experiment

It is often an advantage to be able to study the metabolic response of a tissue to various reactants (e.g. substrates, hormones etc.) using the same sample in order to avoid biological variation. A procedure has been worked out, for the 10^{-2} $\mu\text{l/h}$ version where addition of a reactant can be made maintaining the same sample incubation chamber and hemoglobin concentration thus also minimizing technical errors. Drilled glass slides were used, but sapphires of the larger dimension can be used as well. The procedures prior to the introduction of the reactant are identical to those described for these types of measurements above. The recording should be allowed to continue until the increment in absorbance per unit time is strictly constant (between 75 and 50 % oxygen saturation)

The preparation is then removed from the heated object stage, declamped and gently uncovered. The opened chamber including tissue sample is gently flushed with new hemoglobin-containing medium ($\sim 100 \mu\text{l}$) now including the reactant. The flushing is performed under a stereomicroscope to ensure that no sample loss occurs. The preparation is then ready to be covered as described previously though for the case of drilled object slides a slightly smaller cover glass than that used the first time is applied to avoid contact with the crystallized material left at the border of that slide. The procedures are then similar to above and the recording is continued until complete deoxygenation occurs. The relative difference in "basal" and reactant induced respiratory activity can be directly read from the slopes of the linear part of the absorbance shift curves, and absolute respiratory rates are calculated as above. A prerequisite for this type of measurement is that the mixing (re-oxygenation) *per se* does not influence the O_2 -uptake of the tissue subjected to investigation. This is easily checked by running controls, *i.e.* without changing the composition of the medium for the second incubation.

General discussion

Similarities and dissimilarities to other microrespirometric methods with reference to O_2 -tension.

It should be recognized that measurement of oxygen consumption is performed under drastically lower O_2 tension than is generally the case with the manometric procedures and in most cases with polarography. This is due to the inherent principle of oxyhemoglobin dissociation in this system. To some degree the O_2 tension during "measurement" can be changed by adding ligands, such as 2,3 diphosphoglycerate or inositol hexaphosphate to the solution thereby increasing the half saturation value or by complete "stripping" of the pigment thus lowering the P_{50} value (Berman *et al* 1971). It has been shown that the hemoglobin solutions used in these experiments can easily attain an increased P_{50} -value (from 10–15 to 25 mm Hg) on addition of inositol hexaphosphate at hemoglobin equimolar concentrations (Hultborn unpublished).

It is important to consider the relatively low oxygen tension in the medium during measurements, with respect to oxygen gradients within the tissue sample or cell studied. Such gradients occur in solid tissue samples especially when respiratory rates are high and the samples are large. This might cause an inhomogeneous O_2 -supply resulting in hypoxia in the centre of the tissue sample during the latter part of the experiment when the O_2 tension is getting lower. Thus when estimating the respiratory rate of a sample under conditions, too low values as to the true respiratory capacity of the whole tissue are obtained. Herlitz and Hultborn (1974) found in a study of the respiratory activity of solid samples of the corpus luteum from the rat, that the presence of the substrate succinate (inducing a high respiratory rate) resulted in a markedly asymptotic approach to the final "reduced level" of the OD-curve especially when large samples were used ($\sim 20 \mu g$) but a much less pronounced asymptotic approach was noted in incubation experiments of such large samples when no substrate was present i.e. resulting in a fairly low respiratory rate (Fig. 19). Small deviations from the theoretically expected final part of the OD-curve was found in a study of the respiratory activity of isolated Deliers nerve cells, Fig. 5 (Hultborn and Hydén 1974). The deviations were similar to those obtained in an investigation on the O_2 uptake of brain homogenates (Hultborn and Jarlstedt 1974) but homogenates of corpus luteum presented extremely small deviations from the theoretically expected curve (Herlitz and Hultborn unpublished). These observations might reflect a different susceptibility to low O_2 tensions of different tissues.

A recent study by Starlinger and Lubbers (1973) on the respiration of mitochondria revealed that lowering of O_2 tensions down to 0.05 mm Hg did not affect oxygen uptake in contrast to previous reports where conventional O_2 -electrodes were used (e.g. Lubbers 1968, Schuler and Kreuzer 1969). These latter studies indicate a decrease in respiratory rates in the range of a few mm Hg. The explanation for this discrepancy is the considerable inertia of the conventional O_2 -electrodes (Starlinger and Lubbers 1973).

It might be claimed that the microrespirometric system presented here to a greater

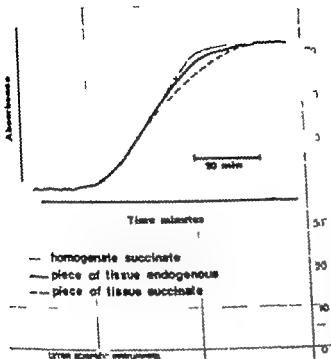


Fig. 19 OD-curves under three different conditions. The middle curve is an actual recording, but the two others are reconstructed from other experiments. Observe the different approaches to the final reduced level indicating different degrees of cellular hypoxia. (From Herlitz and Ilbourn 1974).

extent mimicks the physiological situation *in vivo* regarding oxygen tension than do the manometric procedures, almost invariably performed at atmospheric O_2 tension. This is of course only true when using small tissue samples or isolated cells not causing significant gas gradients.

In relation to oxygen tension and gradients within the medium in the chamber it should be noted that several studies have shown that hemoglobin promotes oxygen diffusion in liquids (e.g. Kluge *et al.* 1956 Scholander 1960 Soell 1965 Wittenberg 1965). Further no differences of the degree of saturation of the hemoglobin at different distances from the sample have been noted in the incubation chambers in this investigation. A rapid equilibration of oxygen within the medium can therefore be assumed.

Other pigments reversibly associated with oxygen can also be used as indicators of O_2 -tension. For myoglobin however the P_{50} value is considerably lower than for hemoglobin, making it less suitable for routine microrespirometry. However certain animal blood pigments (cf Altman and Dittmer 1971 Moll 1972) have high P_{50} values but the use of human hemoglobin is convenient, since it is easily obtained.

Simultaneous to oxygen fixation in the tissues a continuous production of carbon dioxide takes place. In the manometric procedures an alkaline solution absorbs the liberated CO_2 , but this cannot be done in the polarographic or spectrophotometric procedures. The alkaline fixation of CO_2 in the manometric technique is used to avoid disturbance of the volumetric measurements. It can be calculated that in the closed system adopted in the spectrophotometric technique using an adequately buffered incubation, no significant change in pH due to carbon dioxide will occur during the half-saturation and the mere presence of hemoglobin potentiates the buffer.

Oxygen consumption rates suitable for studies with the spectrophotometric method cover a wide range from $1 \mu\text{l O}_2/\text{h}$ to $10^6 \mu\text{l O}_2/\text{h}$. The sensitivity can be varied on the one hand by changing the amount of oxygen available in the system *i.e.* by alteration of the chamber volume and hemoglobin concentration, and on the other hand by changing the amplitude of the OD-curve *i.e.* by variations of the hemoglobin concentration optical pathlength or wavelength for recording. For maximum sensitivity and resolution the volume of the chamber should be decreased as much as possible most efficiently done by reducing the diameter of the cylindrical chambers. Further a wavelength combining low transmission for hemoglobin with a pronounced relative difference in absorbance between the oxygenated and deoxygenated form should be chosen.

In general terms the resolution of the system treated here can be expressed as volume oxygen consumed per unit optical density change the resolution increasing with decreasing values of this ratio *i.e.* It is desirable to get a large change in OD for a small change of oxygen within the system. Due to the presence of physically dissolved oxygen, which is constant at a certain oxygen tension, an increase in hemoglobin concentration, leading to a proportional increase in amplitude of the OD-curve, will not result in a proportional increase in total oxygen content (chamber volume and optical pathlength kept constant). Thus the volume oxygen per unit OD change (dotted curves in Fig. 20) will decrease as the hemoglobin concentration is raised implying an increased resolution. These considerations suggest the use of very high hemoglobin concentrations, which would result in greatly prolonged times for completion of the OD-curves, due to the amount of oxygen in the system. It was therefore calculated how much is

¹ in resolution on raising the hemoglobin concentration and in Fig. 20 it should be derived that hemoglobin concentrations (tetrameric) above 10^{-4} M do not add much to resolution. In Fig. 20 the ratio has been plotted against hemoglobin concentration at two different oxygen tensions of the system, namely at 150 mm Hg (atmosphere) and at 50 mm Hg, the hemoglobin being assumed to be fully saturated with oxygen at both these tensions, which is approximately "true" for the hemoglobin solutions used in this investigation. The ratio has lower values at the lower oxygen tension since the proportion of hemoglobin-bound O_2 to physically dissolved O_2 has increased due to the decrease of the physically dissolved pool. The ratio will not decrease much more at further lowering of the O_2 tension since the oxyhemoglobin will then progressively dissociate.

In this context it should be emphasized that completion of the OD-curve in each experiment is not necessary since the ratio of the volume O_2 per unit change in OD is known as discussed above. Therefore only a small section of the linear part of the OD-curve has to be recorded, a procedure partly applied by Herlitz and Hultborn (1973) in mixing experiments. Such a procedure significantly increases measuring capacity since time of measurement can be kept very short, especially if the preparation is flushed with a gas mixture having an oxygen tension of 20–50 mm Hg before closing the chamber thereby reducing the amount of physically dissolved oxygen.

The resolution was above defined as the ratio of the volume consumed oxygen per unit change in optical density. However various detecting instruments have different capacities to amplify a certain OD-change to make it measurable at the data output level. The signal to noise ratio of the detecting equipment generally does not play any role since the time

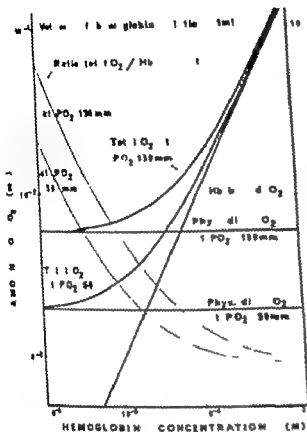


Fig. 11 Illustration of the amount of hemoglobin-bound and physically dissolved oxygen and the sum of these with changing hemoglobin concentration. The situation is illustrated for two different O_2 tensions the hemoglobin being assumed to be fully saturated with oxygen in both. The expression for the resolution of the method the ratio of the volume O_2 per unit change in optical density is illustrated by the dotted curves in the figure. - OD is replaced by Hb-concentration in they are directly proportional to each other. It is seen that not much is gained in resolution on increasing the hemoglobin concentration above $10^{-4} M$. A slight decrease in amount of physically dissolved oxygen with increasing hemoglobin concentration is disregarded. For further discussion see the text.

constant of the kinetic reaction of the object is so large that adequate damping can be used. For single beam instruments it is necessary to test the long time stability so that no drifts in signal occurs.

Another way of increasing resolution of the system and thereby the sensitivity is to use a reflecting microscope and thus have the measuring light-beam reflected at the bottom of the chamber. The optical pathlength is then doubled and an increased change in optical density per volume oxygen consumed is achieved.

The reproducibility of the system as used in this investigation is dependent on the constancy of the various parameters included, such as accurately determined chamber volumes and hemoglobin concentrations. The constancy of the oxyhemoglobin kinetics is of importance both for the true respiratory rate of the biological material studied (a drastic decrease in half-saturation value could decrease respiratory rate) and for the calculations on the obtained OD-curve. Hultborn *et al* (1974) using the $1 \mu l O_2/h$ version found from an analysis of ten experiments with a common yeast cell suspension reproducibility of $\pm 1\%$ (1SD) for the instrumental system together with the dissociation kinetics of the hemoglobin. Herlitz and Hultborn (1974) used the $10^{-2} \mu l O_2/h$ modification found in a study of respiration of corpuscles leading to significant central anoxia of the sample were present to add to the variation however a considerable error of weight measurement. The biological variation. In conclusion it seems that the reproducibility of the system is high.

spectrophotometric system is the same as or even better than the micromanometric techniques (Herlitz and Hultborn 1974)

Other considerations specific for the spectrophoto-respirometric system.

The system investigated contains a protein as indicator of oxygen tension. The inherent presence of a protein in the system must be considered as a potential risk of interference with the metabolic events subject to study. A direct "toxicity" to the tissue must be ruled out as well as interference with substrates hormones etc. present in the incubation medium. Hultborn (1972) made a dose response study of yeast cell respiration varying the hemoglobin concentration from 5×10^{-6} to $1 \times 10^{-3} M$ without finding any significant covariation in respiratory rates. Bärzu and Borza (1967) did not find any relation between hemoglobin concentration and the oxidative activity of isolated mitochondria. Further Herlitz and Hultborn (unpublished) performed standard diver respirometry on samples of corpus luteum in the presence of $5 \times 10^{-6} M$ oxyhemoglobin in the medium of the diver without finding any difference in respiratory rates from control divers without hemoglobin. These data substantiate the suggestion that hemoglobin generally does not influence on tissue metabolism. In the diver experiments mentioned above succinate was used as substrate and the action of this was obviously not interfered with by the protein present.

Since temperature is a parameter on which metabolic activity is highly dependent it was investigated whether the measuring beam, due to its partial absorption in the medium, could raise the temperature of the sample. The energy flux through the object was measured by a calibrated photomultiplier tube the cathode placed near the object plane but any object in the path of the light beam. Assuming complete absorption of the energy in the medium calculation of the "heat" dissipation through the material surrounding the chamber discloses that a completely insignificant increase in temperature occurs in the chambers due to energy absorption from the incident light beam.

Since it is of prime importance that no gas exchange occurs between the chamber and the environment it was tested that a hemoglobin solution deoxygenated by nitrogen flushing was not reoxygenated within the closed chamber. It was also tested that an oxyhemoglobin solution in a closed chamber was not transformed to carboxyhemoglobin when the whole preparation was situated in a carbon monoxide environment.

Facility and versatility

In a study comparing the classical "standard-diver" technique and the spectrophotometric one (Herlitz and Hultborn 1974) it was stated that the whole procedure was considerably more convenient using the latter method. By use of automatic cuvet changers the capacity of measurements is obviously increased. The automatic recording involved in spectrophotometry is also advantageous over the discontinuous manual determinations most oftenly used in micromanometry. Without any changes of equipment except for the chambers respiration rates from 10^{-2} to $10^{-6} \mu l O_2/h$ can be measured.

The microspectrophotometric equipment used for this type of investigation can without any alterations be used for other purposes than microrespirometry such as ordinary microscopy microphotography cytophotometry etc.

Applications

The microrespirometric system described in this work has been applied to various tissues under different conditions. Below a brief review of these studies will be given.

The $1 \mu\text{l O}_2/\text{h}$ version suitable for measurements on cell suspensions or tissue homogenates was used by Hultborn (1972) in a study of yeast cell respiration at various concentrations of hemoglobin in the medium (see above). Hultborn and Ölling (1973) investigated the metabolic properties of leucocyte enriched fractions from normal blood-donors and from individuals suffering from chronic granulomatous disease (CGD). Normally granulocytes exhibit a marked increase in respiratory activity (respiratory burst) on stimulation to phagocytosis, but cells from patients suffering from CGD lack this burst which was easily demonstrated by use of the differential-spectrophotorespirometry (Fig. 10). Further the respiratory rates of tissue homogenates from brain and liver (Hultborn and Jarlstedt 1974) and from the corpus luteum of the rat (Hertitz and Hultborn unpublished) were investigated.

Investigations utilizing the microscope spectrophotometer equipment include a study of the respiratory activity of small samples of well defined corpus luteum from the rat (Hertitz and Hultborn 1974). The influence of different substrates on the O_2 -uptake was studied and the results were compared with those obtained in experiments using classical standard-drivers. Further (Hertitz and Hultborn 1973) reported successful addition of substrates in the course of an experiment as well as the use of the automatic carrier-changer. Hultborn and Hyden (1974) studied the metabolic response to increased potassium concentration of isolated Deltic nerve cells (Figs 5-11) where a profound increase in O_2 -uptake in the presence of 50 mM KCl as compared to 4 mM KCl was found. The oxygen consumption of small numbers of yeast cells (5-20 cells) were determined by Hultborn (1972) who used an early version of the ultrasmallTM incubation chambers suitable for oxygen uptakes in the range of $10^{-4} \mu\text{l/h}$. The sapphire jewel chambers suitable for this range of work have been tested using yeast cells (Hultborn unpublished).

Investigations are in progress (Hultborn and Rockert unpublished) where capillaries (internal diameter 0.4 mm, length 30 mm) are filled with solutions of brain homogenate at a pressure of 6 atmospheres (air) the homogenate suspension being equilibrated with air at this pressure beforehand. As expected, the time elapsed before the start of the dissociation of the oxyhemoglobin was prolonged. This indicates the possibility to use this system for determination of oxygen dissociation curves by an approach related to that published by Longmuir and Chow (1970) though in their study oxygen tension is traced instead of hemoglobin saturation.

Assessment of cellular and tissue metabolism has not been commonly used for functional categorization of oncologic and metabolic diseases (cf Warburg 1962). This situation is due to the hitherto wellknown complexity and difficulty of such analyses. The relative simplicity of the method presented in this work may however open new possibilities for such functional studies in combination with cytological and histological examinations on material obtained by different types of biopsies. This would introduce a new dimension in classification of neoplastic diseases, and furthermore it permits testing of potential therapeutics *in vitro*.

Summary

- 1 A method is presented suitable for measurements of O_2 -uptakes between $1 \mu l/h$ to $10^{-6} \mu l/h$.
 - 2 The sample subject to study is incubated in a closed chamber (cuvet) in a medium containing oxyhemoglobin. The gradual deoxygenation of the hemoglobin, due to the sample respiration, is recorded spectrophotometrically at 435 nm.
 - 3 The theoretical background of this system as well as an analysis of the resolution of the system are presented.
 - 4 The relatively low oxygen tension at which measurement of respiration is performed using this technique is discussed in relation to manometric measuring systems and to the situation *in vivo*.
 - 5 A dual-beam spectrophotometer was adapted for assessment of oxygen consumption of cell suspensions and homogenates in the range of $1 \mu l O_2/h$. To increase measuring capacity a cuvet-changing device was developed.
 - 6 For determinations of respiratory rates of solid tissue samples or isolated cells a single-beam microscope spectrophotometer was used. Incubation of samples was made in glass or sapphire cuvetts of minute dimensions. The sensitivity was brought to the range of $10^{-6} \mu l O_2/h$.
 - 7 The versatility, facility and accuracy of this method were compared with those of a micromanometric procedure. It was found that the microspectrophotometric respiration was more easily operated than was the diver procedure. The accuracy was as high as or even better than that for micromanometry.
- Brief notes on investigations applying the spectrophoto-respirometric procedure to various biological materials, such as blood cells, brain and ovarian tissues are given.

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The effect of quantitated training on the
capacity for short and prolonged work

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ABSTRACT

NORDESJÖ L.-O. *The effect of quantitated training on the capacity for short and prolonged work.* Acta physiol. Scand. 1974 90 Suppl. 405

A group of 27 subjects followed individual training programs on the bicycle ergometer for 8 weeks. The training programs were varied as to the intensity (50 75 or 100 % of the work rate that could be sustained the set time), frequency (1 3 or 5 times per week), and duration (15 60 or 120 minutes). After 4 weeks training, adjustments based on the increases in work capacity were made for the individual work rates.

Analysis of the results showed that the most important factor was the intensity of training, but frequency and duration could also be shown to be of significant importance. Some improvement of both short ($W_{max 6 \text{ min}}$) and prolonged ($W_{max 90 \text{ min}}$) work capacity was achieved even with the easiest program, but the greatest improvements followed training with maximum intensity frequency and duration. There was no significant relationship between initial work capacity and improvement, even if the influence of the intensity frequency or duration of training was eliminated.

LIST OF SYMBOLS AND ABBREVIATIONS

\bar{x}	a bar over a term denotes a mean
b	slope of regression line
b_n	slope of regression line at specified round of test
CH	chest-head lead
CR	chest-right arm lead
CPW	capacity for prolonged work (90 min)
CSW	capacity for short work (6 min)
D	duration of training session
ΔCPW	difference in capacity for prolonged work before and after training
ΔCSW	difference in capacity for short work before and after training
DLG	Dauerleistungsgrenze (see section I)
ECG	electrocardiogram
EPS	Erholungspulssumme (see section I)
F	frequency = number of training sessions per week
HR	heart rate (beats \times min ⁻¹)
HR _{max}	heart rate at end of test for short time work capacity
HR _{work}	mean heart rate during training
I	intensity = highest work rate that can be sustained for a given time
LPI	Leistungspulsindex (see section I)
N	work rate (= load) during tests training
N _{pw}	work rate during tests for prolonged work capacity
N _{sw}	work rate during tests for short time work capacity
O ₂ -DLG	Dauerleistungsgrenze according to Hoffmann (the value below which oxygen uptake does not rise, irrespective of the duration of time)
T	time
T _{pw}	work time for test of the capacity for prolonged work
T _{sw}	work time for test of the capacity for short work
V	gas volume per unit time
$\dot{V}O_2$	volume of oxygen per minute (= oxygen uptake per min)
$\dot{V}O_{2\max}$	maximal oxygen uptake
W	work rate
W_{130}	work rate at heart rate of 130 beats per min
$W_{\max \times \text{min}}$	th highest work rate that can be sustained for a given time
Statistical terms	
\bar{x}	mean
d	mean difference or difference between two means
SD	standard deviation
n	number of subjects
s_y	standard deviation of residuals around the regression line
s_y^2	variance in the dependent variable
r	coefficient of correlation
$r_{xy \dots}$	coefficient of partial correlation
R	coefficient of multiple correlation
p	probability
*	probably significant ($p < 0.05$)
**	significant ($p < 0.01$)
***	highly significant ($p < 0.001$)

I INTRODUCTION

The human capacity for physical work varies within very wide limits. An individual's physical work capacity is chiefly determined genetically but can to a great extent be influenced by training as well as by inactivity (Cuthbertson 1929 Taylor *et al* 1949 Saltin *et al* 1968).

There seems to be agreement that physical activity is beneficial for the individual, but as regards the prevention of coronary heart disease, for example, the benefit of physical activity has not yet been proved (Andr e Larsen and Malmborg 1971).

On the other hand, no definitive guidelines on the mode of training can be given. Various types of training, mainly interval, distance and circuit training all have their proponents. In a few studies different types of training have been compared (Clasing *et al* 1966 b Hollmann *et al* 1966 Roskamm *et al* 1966 b). The results were not quite conclusive, but it seemed as if distance training was equally efficient or slightly better than interval training.

Neither can any definitive guidelines be given on the amount of training necessary to give a sufficient increase in physical work capacity. Several of the previous studies on training were of the cross-section type, studies in which athletes and non-athletes were juxtaposed for purpose of contrast. In longitudinal studies, where the subjects in training have been followed for 2-6 months, the maximal oxygen uptake has been reported to increase between 7 and 33 % (Robinson and Harmon 1941 Knehr *et al* 1942 Taylor *et al* 1949 Rowell 1962 Ekblom *et al* 1968 Saltin *et al* 1968 Saltin *et al* 1969 Kilbom 1971 a, b and c).

There is a rather limited number of studies in which the authors have attempted to make an exact quantification of the training (Roskamm *et al* 1966 a and b Siegel *et al* 1970 Kilbom 1971 a and c). In two studies the intensity, duration, and frequency of training have been varied, and the attempt has been made to describe the change in work capacity as a function of the training's intensity, frequency and duration as well as of the initial work capacity (Shephard 1968 Davies and Knibbs 1971).

The above mentioned studies have all dealt with the effect of training on the capacity for short time work (2 to 10 min), either expressed as work rate at a set heart rate (W_{170}) (Roskamm *et al* 1966 a and b), watt pulse (Roskamm *et al* 1966 a and b), or as estimated (Shephard 1968) or measured oxygen uptake capacity (Siegel *et al* 1970 Davies and Knibbs 1971 Kilbom 1971 b and c). A large number of tests have been devised in order to study the capacity for short time work (for references see Hainman 1967 Blohmke 1969 Lange Andersen *et al* 1971).

However from the standpoint of work physiology the capacity for prolonged work ought to be of equally as great an interest. This work capacity has been defined as the work rate an individual can maintain during eight hours without becoming exhausted, i.e., the processes of fatigue and recovery are in equilibrium (Karrasch 1951 M ller 1961). Since in work with large muscle groups it is probably factors which primarily set the limit, M ller and his associates

of the capacity for prolonged work could be obtained by recording the heart rate during a work day. Heart rate was subsequently recorded both during occupational work and during exercise on a bicycle ergometer. It was found that at a certain work rate, which was specific for each individual, the heart rate, after an initial rise, remained constant (increase of less than 1 beat per min per hour Rohmert 1962) whatever the duration of work. The total recovery heart rate (Erholungspulssumme = EPS), i.e. the sum of the heart rate during the period of recovery minus the sum of the heart rate at rest during an equivalent period of time, also remained constant. Müller designated this work rate as Dauerleistungsgrenze (DLG) (Karrasch and Müller 1951).

Subsequent experiments showed that it was possible to determine DLG by means of three or four 10 (Müller 1955) or 60 (Rohmert 1962) minute work tests with different work rates and recording of EPS. During work at the DLG-work rate the heart rate was seldom higher than 110 beats per minute, i.e., seldom more than 33–40 beats above the heart rate at rest (Müller 1961 Rohmert 1962). Since both methods are highly time-consuming, Müller (1950) introduced an index, designated as the Leistungspuls Index (LPI), as a more easily obtained measure. This is based upon the rate of increase in the heart rate when the work rate during an ergometer cycle test is raised every minute by $60 \text{ kpm} \times \text{min}^{-1}$ from 60 to $600 \text{ kpm} \times \text{min}^{-1}$.

In conducting spiroergometric studies, Hollmann (1963) found that during work at certain work rates the oxygen debt was higher after 20 minutes of work than it was after five minutes, while at other and lower work rates it remained the same regardless of whether the work lasted five or 20 minutes. The highest work rate which gave the same oxygen debt after 20 minutes as it did after five minutes was designated O_2 DLG. This work rate is higher than the DLG defined by Müller and consequently results in a greater increase of heart rate. The O_2 DLG is approximately the same work rate as W_{130} .

Is the reason for Hollmann's proposal that endurance capability simply be measured as W_{130} ? In several studies involving military conscripts the mean value of W_{130} has been shown to lie between 600 and $702 \text{ kpm} \times \text{min}^{-1}$ (Clasing *et al.* 1966 a and b Roskamm *et al.* 1966 b).

Müller points out that one ought not to expect any relationship between a capacity for 8 hours of work and maximal oxygen uptake (1961). He bases his statement on observations made in conjunction with a study of a group of subjects which was very heterogeneous with respect to age. But Müller himself showed that there was a relationship ($r = 0.89$ $p < 0.001$) between the group mean values for LPI and maximal oxygen uptake for the subgroups who were 10 to 33 years old. In the original description of the LPI, based on 16 subjects without recorded age, Müller (1950) found a relationship between LPI and W_{max} ($r = 0.71$ $p < 0.01$).

In a study of 15 young men Davies and Harris (1964) have also succeeded in showing a relationship between LPI and maximal oxygen uptake ($r = 0.56$, $p < 0.05$).

On the basis of calculations founded on daily caloric intake, caloric consumption during work, and maximal oxygen uptake, Bink (1962) has shown that there probably is a linear relationship between work time and absolute work rate when both are expressed as logarithms. Furthermore, a direct relationship between maximal oxygen up-

take and oxygen consumption in daily work has been shown for construction workers. In daily work the oxygen consumption was about 40% of the maximal (Åstrand, L. 1967).

Thus, there appears to be a relationship between the capacity for short time work and the capacity for work of longer duration.

In many instances, however work of long duration which results in a certain degree of exhaustion is of greater interest than work at DLG work rate. In these cases W_{130} , as suggested by Hollmann, is a conceivable work rate and a conceivable measure. At a heart rate of 130 beats per min the oxygen uptake is about 50 per cent of the maximal in young individual of about 25 years of age (Åstrand, L. 1960).

However well-trained individuals can sustain a balance between oxygen uptake and oxygen consumption at a higher relative work rate (work rate at which the oxygen uptake is a certain per cent of the maximal) than can untrained individuals (Åstrand and Rodahl 1970). For this reason W_{130} is not entirely suitable as a measure of the capacity for prolonged work. Furthermore, W_{130} like LPI has a relatively low reliability (Blohmke *et al.* 1966 Ehrenstem and Müller-Limmroth 1968 Blohmke 1969).

Rohmert (1962) has shown that there is a linear relationship between log maximal work time and log work rate (expressed as a multiple of DLG) for work within the time interval 8–197 minutes. Ahlborg (1966) studied work of approximately the same duration (2–240 min) and found equivalent correlations between log maximal work time and work rate ($r = 0.90$) and between log maximal work time and log work rate ($r = 0.89$). Consequently it is possible to study the capacity for prolonged work by methods which are designed on the basis of the relationship between maximal work time and work rate.

II PURPOSE OF THE STUDY

The present study proposes to determine

- 1 How the capacity for short time and prolonged work is influenced by training of varying intensity frequency and duration
2. If the relationship between the capacity for short time and prolonged work is changed as a result of training
- 3 To what degree of certainty the capacity for prolonged work can be predicted.

III DEFINITIONS AND TERMS

In this study every work task which leads to exhaustion will be designated as maximal, and the work rate which leads to exhaustion after t minutes will be designated $W_{max\ t\ min}$.

The capacity for short time work is defined as the highest work rate the test subject can sustain for 5 minutes ($W_{max\ 5\ min}$), and the capacity for prolonged work is defined as the highest work rate the test subject can sustain for 90 minutes ($W_{max\ 90\ min}$). The capacity for short time and prolonged work are hereafter abbreviated in the tables and equations as CSW and CPW respectively.

The intensity of a training task is given here as a percentage of the highest work rate the subject manages to sustain throughout the entire training session. If the test subject was completely exhausted by the end of the training session, the intensity has consequently been 100 %. In absolute terms this means that a given intensity for example 75 %, denotes different work rates if the period of training is to be, for example, 15, 60, and 120 minutes respectively (In comparing this study with others, where intensity is defined in other ways, for example as a percentage of $V_{O_{2max}}$, one must keep this definition in mind).

IV CONSIDERATIONS TAKEN DURING THE PLANNING OF THE EXPERIMENT

In as much as the primary interest of this experiment was tied to the effect of training on the capacity for prolonged work, it seemed natural to construct the training programs as so-called distance training i.e. continuous work at constant work rate. One reason for this choice is the fact that in a series of training experiments (Roskamm and Clasing 1967) distance training has yielded an equivalent or even better effect on W_{max} than has interval training.

Since training sessions of long duration could be expected to be more efficient than training sessions of short duration as regards the training of the capacity for prolonged work, it was decided that 2/3 of the training programs would involve training periods of very long duration, while for the sake of comparison 1/3 would be used for shorter periods. The duration of the training sessions was fixed at 120, 60 and 15 minutes respectively.

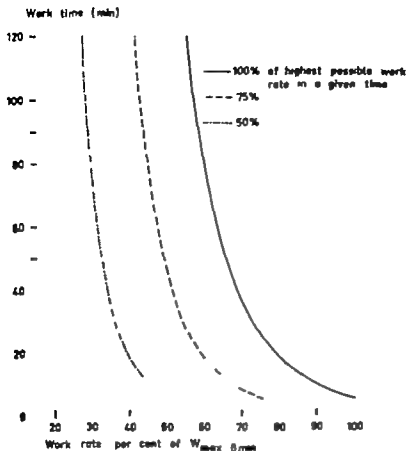
The results of previous studies (Michael *et al.* 1961 Åstrand, I 1967) indicate that an individual manages to work for 8 hours without becoming appreciably tired at a work rate which is the equivalent of 35–40 % of the maximal oxygen uptake. Training at a lower work rate can therefore be expected to have a very low or no effect at all.

By means of the relationship between maximal work time and work rate and that between W_{max} and $\dot{V}O_{2max}$ (Nordesjö 1974 a) as well as Åstrand's nomogram (1960) one can convert the work intensity expressed as work rate in per cent of maximal work rate for a given duration of work, into intensity expressed as oxygen uptake per cent of the maximal oxygen uptake.

An example: assume that W_{max} for an individual is $1500 \text{ kpm} \times \text{min}^{-1}$. His maximal oxygen uptake is then calculated to be about $3.2 \text{ l} \times \text{min}^{-1}$ ($\dot{V}O_{2max} = 0.001685 W_{max} + 0.7208$). If he works at 80% of W_{max} i.e. $1200 \text{ kpm} \times \text{min}^{-1}$ the oxygen uptake should be about $2.8 \text{ l} \times \text{min}^{-1}$ i.e. an oxygen uptake which is about 87.5 % of the subject's maximal oxygen uptake.

According to the relationship between log work time and log work rate as stated by Rohmert (1962) and Tornvall (1963), the highest work rate an individual can sustain for 120 min ($W_{max, 120 \text{ min}}$) corresponds to 55 % of the highest work rate the individual can sustain for 8 minutes ($W_{max, 8 \text{ min}}$). This means that 50 % of $W_{max, 120 \text{ min}} = 27.5 \%$ of $W_{max, 8 \text{ min}} = 37 \%$ $\dot{V}O_{2max}$, which is equivalent to the above mentioned level reported by Michael *et al.* (1961) and Åstrand, I. (1967).

Since it was considered of less interest to train the test subjects at work rates which could hardly be expected to yield any increase in work capacity the lowest intensity was fixed at 50 % of the highest work rates which the test subject could be expected to sustain for 15, 60 and 120 minutes respectively. At the same time, certain test subjects were to train at the highest possible intensity (100%), while other trained at 75%. Fig. 1 illustrated the proportional relationship of the chosen intensities to $W_{max, 8 \text{ min}}$ and $\dot{V}O_{2max}$ for training periods of different duration.



		Work rate per cent		V_{O_2} ml		$\dot{V} W_{\max}$ 8 min	
29	40	48	57	65	73	82	90
	38	46	57	66	77	88	98
	28 36	47 50	57	64	81	91	100

1000 kpm min^{-1}

1500 kpm min^{-1}

2000 kpm min^{-1}

Fig.1 Relationship between work rate as percentage of physical work capacity expressed as W_{\max} 8 min and work time according to the equation $T = 6 \times 10^{-10} (\text{work rate in \% of } W_{\max} 8 \text{ min})^b$ when $b = -5$. The lines represent 50, 75, and 100% of the highest work rate that can be sustained for any given time. At the bottom of the figure is given the oxygen uptake, expressed as a percentage of maximal oxygen uptake, equivalent to the work rate when W_{\max} 8 min is 1000, 1500, and 2000 kpm min^{-1} respectively.

The frequency of training was also arranged in three steps: 1, 3 or 5 times a week. In this way 27 different training programs (see Fig. 2) were obtained, and every subject was to train for 8 weeks according to one of these programs.

Duration of training session (min)	15			60			120		
Frequency of training (times per week)	1	3	5	1	3	5	1	3	5
Intensity of training (%)									
50	0	0	X	0	0	X	0	X	X
75	0	0	0	0	0	X	X	●	X
100	X	X	X	X	X	X	X	X	X

X = Spring 1969

■ = Spring 1970

● = Autumn 1970

Fig. 2. Training programs.

V SUBJECTS

A total of 29 men applied after the announcement of the experiment. The reason for participation in the experiment was the possibility for improved physical fitness as a result of the training. The test subjects were also offered a modest remuneration which covered travelling expenses and also served as an incentive for further participation, in as much as this compensation would only be given out after completion of the training program.

Of the 29 test subjects, 2 were actively engaged in athletic competition and were therefore excluded from the series. One test subject became ill while the tests were in progress, and a replacement underwent the equivalent training program 6 months later.

Data on the 27 test subjects who took part in the whole program are given in Table I. This table shows that the test subjects, whose average age was 22.5 years, were slightly taller (unpublished data) and heavier (Åhlborg *et al.* 1972) than 18 year-old military registrants (Lindroth 1969) but that their capacity for physical work was significantly ($0.05 > p > 0.01$) higher. This was probably because the groups consisted of volunteers.

Twenty-four test subjects were full-time university students, and three others held active but very light jobs. The majority of the university students worked during the summer—6 to 9 months before the beginning of the experiment—in various occupations, ranging from heavier manual labour to desk jobs. Five test subjects were physically inactive even during their spare time: seventeen played tennis, rode, or did some cross-country running on occasion, i.e., were somewhat active, and five subjects were more

TABLE I. Physical characteristics of the subjects ($n = 27$) and some data on physical activity and capacity compared with data for a representative sample of Swedish boys of 18 ($n = 1719-1789$).

	The subjects		Range	Boys of 18
	\bar{x}	SD		\bar{x}
Age, years	22.7	1.8	20.0-25.8	18.5
Height, cm	180.7	6.5	170.5-194.0	177.2
Weight, kg	67.9	8.0	55.2-84.5	66.4
Heart volume, ml	740	113	525-1070	673 ^a
Heart rate at rest, beats \times min ⁻¹	67.7	7.3	54-84	74.9
Physical activity				
occupational	1.1	0.3	1-2	—
recreational	2.0	0.6	1-5	—
W _{max} 6 min	1556	235	1107-2114	1451
kpm \times min				
Heart rate at end of work test, beats \times min ⁻¹	190.9	8.2	174-204	193.6

^aCalculated from chest photo fluorogram taken in the standing position

regularly engaged in physical exercise, in the form of swimming, cross-country running or bicycling. None of them competed actively in athletics, but one had been an active cross-country runner the year before. Nineteen test subjects were non-smokers, although two of them used snuff. Three smoked 1–10 cigarettes a day and five smoked 11–20 cigarettes a day.

Due to limited capacity 16 test subjects were examined in the spring of 1969, 10 in the spring of 1970 and 1 (the replacement for the test subject who became ill in the spring of 1970) in the autumn of 1970.

According to their own reports, the test subjects' physical activity and fitness were basically the same during the 6 months prior to the training study. Fifteen of the 16 test subjects who trained in the spring of 1969 had in the autumn of 1968 participated in experiments regarding method reliability (Nordesjö 1974b). Their own reports can therefore be supplemented by the test results from September and December 1968, and from the period immediately prior to the training. The mean value for $\dot{V}_{\text{max } 6 \text{ min}}$ was 1634, 1647 and 1630 $\text{kpm} \times \text{min}^{-1}$ respectively. Heart volume and weight were entirely unchanged: 728 ml and 67.5 kg, 736 ml and 67.2 kg respectively.

Apart from the fact that one test subject began to train by running somewhat during the last 14 days, the test subjects' physical activity was unchanged both at work and during free time.

VI METHODS

I General

Information regarding the test subjects' smoking habits and physical activity during spare time and while at work, or the equivalent (studies), was obtained by means of a questionnaire based upon one which has been used previously in studies of training (Saltin and Grimby 1968 Kilbom *et al.* 1969). Smoking was registered in terms of the number of cigarettes per day and physical activity was graded according to the following.

Physical activity during spare time

- 1 Almost completely inactive reading, TV movies, etc.
- 2 Some physical activity during 4-6 hours a week riding a bicycle or walking to work, walking with the family fishing, working in the garden, playing table tennis, bowling
- 3 Regular physical activity such as running, calisthenics, tennis, swimming
- 4 Regular hard physical training several times a week for competition in running events, soccer handball, etc.

Physical activity while at work or the equivalent

- 1 Predominantly sedentary work studies, desk work, watchmaking, sitting assembly worker (light goods)
- 2 Sitting or standing work, some walking shopclerk, office worker light tool and machinery worker foreman, teacher or instructor
- 3 Walking and some handling of material mailman, waiter construction worker heavy tool and machinery worker
- 4 Heavy manual work lumberjack dockworker stone mason, farmworker unskilled labourer fisherman.

Height was taken to the nearest 0.5 cm, with the test subject standing without shoes.

Weight was taken to the nearest 0.1 kg, with the test subject clad only in gym shorts.

ECG was registered at rest and after 8 minutes in standing position with 11 leads on an Elema mingograph 42 or 34. The speed of the paper was checked by measuring the length of the paper during a set period of time. At least 20 complexes were registered by ECG during work. At rest and while standing leads CR_{1-3} were registered and during the initial work test leads CH_{2-7} . During the subsequent work test and training sessions the heart rate was registered by means of lead CR_4 .

Heart volume was calculated according to the formula proposed by Kjellberg *et al.* (1951) from antero-posterior and lateral roentgenograms taken with the tube at right angles to the table. The subject was placed in a supine position with cranially extended arms. No consideration was paid to the phase of the heart cycle. Owing to technical reasons roentgenograms were not taken on all subjects before and after training. On

those that were, there was no significant difference ($d = + 17.46$ ml, $p > 0.2$, $n = 16$) between the two results, and no relationship could be discerned between the difference in heart volume on the one hand and the frequency intensity or duration of the training on the other. Since the difference was so small the mean of the individual values before and after training are taken to represent each subject's heart volume, except for the cases where there only exists one value.

2. Bicycle ergometers

Tests and training were conducted on 2 slightly modified electrically braked bicycle ergometers, Elema-Schölander model AM 368 (Holmgren and Mattson 1954). The calibrations of the bicycle ergometers were checked at the factory before, during and after each of the test series (spring 1969 spring 1970 and autumn 1970). No deviations from the calibration report were noted in so doing. During the standard calibration procedure the ergometer is run in the test bench for about 10 min at a low load. A nominal load (= work rate) at $50 \text{ kpm} \times \text{min}^{-1}$ is then set and after 1 min the torque at 60 r.p.m. is recorded on a balance and the actual load is calculated. The nominal load is then increased to $100 \text{ kpm} \times \text{min}^{-1}$ the torque recorded after 1 min and so on until 2000 or $2050 \text{ kpm} \times \text{min}^{-1}$. The procedure takes 17 min and is then repeated for 45 and 75 r.p.m.

As can be seen from Table 2, the actual load falls below the nominal load during prolonged work on higher loads. Before modification the actual load was about $30 \text{ kpm} \times \text{min}^{-1}$ below the nominal $1800 \text{ kpm} \times \text{min}^{-1}$ after 10 min work, and $70 \text{ kpm} \times \text{min}^{-1}$ below nominal after 60 min work.

In as much as the highest measureable work rate on ergometer model AM 368 is $2050 \text{ kpm} \times \text{min}^{-1}$ a test subject with a very high initial work capacity had to perform tests and training on ergometer cycle model 361. On this model the actual work rate increases during work performed at high levels (see Table 2). The values of the above mentioned test subject have been corrected on the basis of the results of trials in the test bench on the work rates which were used during tests and training.

On both ergometer the pedal length was fixed at 17.5 cm. The saddle height was adjusted in such a way that the test subjects' legs would be flexed about 5 degrees at the lowest point of revolution.

3. Determining the capacity for physical work

All work tests were conducted without warming-up and continued until the test subject could no longer maintain the prescribed number of revolutions, 60 r.p.m. At the end of the short time work tests the test subject was completely exhausted and had a high heart rate. However during the prolonged work tests it was more difficult for the examiner to determine whether the test subject was completely exhausted or not. In some

TABLE 2. Deviations from indicated loads in $\text{kpm} \times \text{min}^{-1}$ at 60 r.p.m. for the ergometers used in the present study

Ergometer model 361 No 103					368 Modified 563				666
Nominal load	500	1000	1500	2000	500	1000	1500	2000	1500
Time min									
0	0	0	0	-20	5	10	0	0	0
5	3	11	13	-3	-2	-5	-3	-30	-20
10	6	17	23	8	-5	-15	-7	-40	-30
15	7	25	26	18	-6	-20	-25	-35	-30
20	7	25	33	40	-8	-20	-25	-30	-30
30	8	28	53	65	-8	-25	-25	-30	-30
40	8	28	68	100	-8	-25	-25	-20	-30
50	8	32	78	135	-10	-25	-25	-20	25
60	8	32	83	170	-10	-25	-30	-15	-25
70	8	40	93	200	-10	-25	-30	-10	-25
80	10	40	103	220	-10	-25	-30	0	-5
90	10	40	108	235	-10	-25	-30	+10	-22
100	10	46	108	255	-12	-25	-30	+10	-22
110	10	46	-	280	-12	-25	-30	+15	-22
120	10	49	-	230	-12	-20	-25	+20	-22

cases the test subject appeared to be completely exhausted, in others the heart rate was nearly or equally as high as it was during any of the subjects short time work tests. Again, in other cases where the test subject still had a moderate heart rate the test subject broke off the test with the explanation that his legs could take no more. In doubtful cases a new test was carried out a few days later. Both during the prolonged work tests and during training the test subject was allowed to drink *ad lib*. The amount of water consumed (50–1500 ml) varied with the intensity and duration of the work and room temperature. Loss of water due to sweating and ventilation were, however not fully compensated. For example, during 60 min of training with 100 % intensity at a room temperature of 21°C the subject drank 440 ml of water but lost 0.4 kg in weight. The following work tests were used.

1. During the preliminary study a work test was used in which the work rate was initially 300 or 600 $\text{kpm} \times \text{min}^{-1}$ and was raised by 300 $\text{kpm} \times \text{min}^{-1}$ every 6 minutes until exhaustion occurred. A value of $W_{\max 6 \text{ min}}$ was calculated by weighing together the times for the different work rates in terms of a formula stated by Tornvall (number 1 below) according to principles presented previously (Nordenfjöld 1974 b).

2. In determining the capacity for short time work (CSW) the work test of Tornvall (1963) was used, i.e., a work test with constant high work rate where the work time is allowed to vary between 1 and 18 min. The result ($W_{\max 6 \text{ min}}$) is calculated according to the following formula

$$\log W_{\max 6 \text{ min}} = \frac{\log T_{\text{SW}} - \log 6}{-b} + \log N_{\text{SW}} \quad (1)$$

those that were, there was no significant difference ($d = + 17.46$ ml, $p > 0.2$, $n = 16$) between the two results, and no relationship could be discerned between the difference in heart volume on the one hand and the frequency intensity or duration of the training on the other. Since the difference was so small the mean of the individual values before and after training are taken to represent each subject's heart volume, except for the cases where there only exists one value.

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As can be seen from Table 2, the actual load falls below the nominal load during prolonged work on higher loads. Before modification the actual load was about $30 \text{ kpm} \times \text{min}^{-1}$ below the nominal $1800 \text{ kpm} \times \text{min}^{-1}$ after 10 min work, and $70 \text{ kpm} \times \text{min}^{-1}$ below nominal after 60 min work.

In as much as the highest measurable work rate on ergometer model AM 368 is $2050 \text{ kpm} \times \text{min}^{-1}$ a test subject with a very high initial work capacity had to perform tests and training on ergometer cycle model 361. On this model the actual work rate increases during work performed at high levels (see Table 2). The values of the above mentioned test subject have been corrected on the basis of the results of trials in the test bench on the work rates which were used during tests and training.

On both ergometer the pedal length was fixed at 17.5 cm. The saddle height was adjusted in such a way that the test subjects' legs would be flexed about 5 degrees at the lowest point of revolution.

3. Determining the capacity for physical work

All work tests were conducted without warming-up and continued until the test subject could no longer maintain the prescribed number of revolutions, 60 r.p.m. At the end of the short time work tests the test subject was completely exhausted and had a high heart rate. However during the prolonged work tests it was more difficult for the examiner to determine whether the test subject was completely exhausted or not. In some

$$b = \frac{\log T_{PW} - \log T_{SW}}{\log N_{PW} - \log N_{SW}} \quad (4)$$

T_{PW} = work time in min during the prolonged work test

T_{SW} = work time in min during the short time work test

N_{PW} = work rate in $\text{kpm} \times \text{min}^{-1}$ during the prolonged work test

N_{SW} = work rate in $\text{kpm} \times \text{min}^{-1}$ during the short time work test.

(b) with a weighted mean value for the coefficient of regression for the 27 test subjects on each test occasion. This value is calculated according to the following formula

$$\bar{b} = \frac{\sum_{i=1}^m \sum_{j=1}^n (\log N - \overline{\log N}) (\log T - \overline{\log T})}{\sum_{i=1}^m \sum_{j=1}^n (\log N - \overline{\log N})^2} \quad (5)$$

where m = the number of individuals, n = the number of observations per individual, T = work time, and N = work rate (see Snedecor and Cochran 1967 pp. 435 and 443).¹

Since the difference between these two methods of calculating is very small (see the discussion), only the results of method b) are presented in the following.

4 Calculating the work rate for training

In calculating the work rate at which the test subject should be training the results of both short time and prolonged work tests were utilized. By inter or extrapolation it was possible to calculate the highest work rate the test subject could manage to sustain throughout the entire training session, i.e., in 15, 60, or 120 minutes respectively. The test subject was to train at 50, 75 or 100 % of this work rate depending upon the training program he had been assigned to. (In practice, inter or extrapolation was effected in such a manner that the coefficient of regression calculated for the individual was introduced in equation (4) together with the logarithms for work time and work rate in the test of short time work capacity and T_{PW} was replaced by the length of the training period—15, 60, or 120 min. Consequently the unknown factor which can now be calculated will designate the highest work rate the test subject can sustain for 15, 60, or 120 minutes.)

¹ In the case where the individual's regression is based upon two points (2), equation 5 can be simplified to

$$b = \frac{\sum \log T_{PW} - \sum \log T_{SW}}{\sum \log N_{PW} - \sum \log N_{SW}}$$

5 Statistical methods

Current statistical methods were used (Snedecor and Cochran 1967).

To describe a variable the mean value (\bar{X} , \bar{d} or \bar{y}), the standard deviation (SD), and sometimes the minimum and maximum value (here called range) were used.

Simple linear correlation coefficients (r) have been used to describe the association between two variables.

Linear regression analysis was used to describe one variable (y) as a function of one or more other variables (x or x_1, x_2, \dots, x_k) i.e. $y = a + b x$ or $y = a + b_1 x_1 + b_2 x_2 + \dots + b_k x_k$ where a and b are regression coefficients. In the text b is sometimes called slope.

In connection with these functions (sometimes mentioned as equations) the residual standard deviation (s) and multiple correlation coefficient (R) is given. The residual standard deviation is the square root of that part of the variance of y (s_y^2) that is not accounted for by the function. The square of the multiple correlation coefficient equals the fraction of the variance of y that is accounted for by the function.

Sometimes partial correlation coefficients ($r_{12.3}, r_{12.34}$ etc.) are also given. The partial correlation $r_{12.34}$ is the correlation between variables 1 and 2 after elimination of the influence of variables number 3 and 4.

The hypothesis tested is always a null hypothesis, meaning that there is no difference (alternatively correlation etc.) in the (hypothetical) population.

The following table gives the different levels of significance that were used, how they are denominated in the text and how significant correlations, differences, variables etc. are marked in Tables 3–6, 11 and 14.

Probability (p) of rejecting the hypothesis when it is true	Denomination in the text	Mark in tables
< 0.001	Highly significant	***
< 0.01	Significant	**
< 0.05	Probably significant	*
≥ 0.05	Not significant	(no mark)

In treating the data, a stepwise regression program, BMD 02R, University of California 1971 based upon Elfroymsen (1960), has been used in order to obtain correlations and regressions.

VII EXPERIMENTAL PROCEDURE

During a preliminary visit the test subject was to fill in a questionnaire regarding health and physical activity and undergo a general examination which covered a record of individual height, weight, sedimentation rate, Hb, heart volume, blood pressure at rest, ECG at rest, an orthostatic test as well as a simple physical examination, the purpose of which was to eliminate cardiopulmonary disease and the presence of upper respiratory tract infections. After this a preliminary work test was conducted. (Regarding the outline of the test, see section VI. 3.1).

Once the examination showed that nothing hindered the test subject from participating in the study he drew lots (without replacement) and received his assignment to one of the 27 training programs (see Fig. 2).

A few days after the preliminary examination the initial short time work capacity (CSW) was determined (see Fig. 3). Here the test subject was to work at a work rate

Day 1	- Preliminary tests and graded work test	
2	- Determination of heart volume	
etc	- Test on the capacity for short time work	(CSW ₁)
	-	
	- Test on the capacity for prolonged work	(CPW ₁)
	-	
	4 week training	
	-	
	Test on the capacity for short time work	(CSW ₂)
	-	
	- Test on the capacity for prolonged work	(CPW ₂)
	-	
	-	
	4 weeks training	
	-	
	- Determination of heart volume	
	Test on the capacity for short time work	(CSW ₃)
	-	
	Test on the capacity for prolonged work	(CPW ₃)

which was consistent with the result of the preliminary work test. However the work rate was rounded off so as to be divisible by 50

Two days after the test of short time work capacity the initial prolonged work capacity (CPW₁) was determined. (Regarding the outline of the test and choice of work rate, see section VI.3.3).

After another two days time training began at the frequency, duration, and intensity which had been determined for the test subject by the lottery process.

The training continued for four weeks without changes in the work rates. To the extent that work capacity increased during these four weeks the intensity of the training was relatively decreased, in as much as the absolute work rate for training remained constant. In order to determine possible changes in the work capacity and to be able to adjust the work rates for training in such a way that the intensity was once again intended, a second test of short time (CSW₂) and prolonged work capacity (CPW₂) was conducted 2 and 4 days respectively after the last training session in the fourth week of training.

Since it was expected that the test subjects work capacity had increased, a work rate which was somewhat higher than the work capacity measured before the beginning of training (CSW₁) was used during the second test of short time work capacity (CSW₂). (For the test subject who had the hardest training program—100 %, 120 min and 5 times a week—the work rate was about 200 kpm x min⁻¹ higher for the test subject with the easiest program—50%, 15 min and 1 time per week—the work rate was about 50 kpm x min⁻¹ higher)

The appropriate work rate for the second test of prolonged work capacity was calculated according to equation (2), see section VI.3.2, on the basis of work time and work rate from the second test of short time work capacity and on the individual coefficient of regression, which was calculated from the tests which preceded training (b₁).

After this the test subject trained for four more weeks at the work rates which were calculated on the basis of the results from the second tests of short time and prolonged work capacity. Two and four days respectively after the end of training, short time (CSW₃) and prolonged work capacity (CPW₃) were once again determined. The work rates for these tests were determined in the same way as above for CSW₃ 50–200 kpm x min⁻¹ were added to the midway value of short time work capacity (CSW₂), and for CPW₃ the work rate was based upon work rate and work time for the third test of short time work capacity and the slope obtained at the midway test (b₂). However during the last test of prolonged work capacity (CPW₃) the test subject was set to work at a work rate which was about 50 kpm x min⁻¹ higher than had been calculated. The reason for this was a desire to counteract any conscious or unconscious tendency on the part of the test subject to carry out an exactly 90 min long work test. The average work time was also 13 min shorter than during the first test occasion, and this corresponds rather exactly to the difference between estimated and applied work rate.

The method of procedure used here—one which meant that the work rate during short time work tests was chosen with respect to the result of the preliminary test and increases in work rate according to a set pattern—yielded the desired results. The

average work time in minutes was 6.0 (SD 1.5 range 3.4–10.9), 8.8 (SD 3.6, range 4.9–16.5), and 7.1 (SD 3.0, range 4.1–15.4) in tests before, during, and after training. The two, very long work times during the second and third test occasions resulted from the fact that one test subject had to work at a lower work rate than was desirable, since the highest work rate which could be registered on ergometer AM 368 was 2050 kpm \times min⁻¹.

The procedure also led to the desired results during the prolonged work tests. The average work times in minutes were 92.5 (SD 28.3 range 47.2–151.6), 95.3 (SD 38.7 range 50.3–206.2), and 79.3 (SD 19.0 range 50.0–124.0) in tests before, during, and after training.

The intention was to carry out tests of short time and prolonged work capacity during the morning hours and, where possible, at the same time for every individual. This was not entirely successful, in as much as the test subjects had other important activities.

The whole series of tests took a total of 10–12 weeks to complete. As a result of the very long period of examination and training, which did not come to an end until during the summer it was not entirely possible to control the room temperature. On most of the test occasions the temperature was between 20 and 24°C ($22.5 \pm$ SD 1.0), but during the last prolonged work test in the spring of 1969 the temperature was often 24–25°C ($23.9 \pm$ SD 1.2). During the first half of the training period the temperature was approximately 21°C, but during the last two weeks of training the average temperature was 24.9°C (spring, 1969) and 23.3°C (spring, 1970). An electric fan was used in the room when the temperature rose above 22°C.

During the heat waves the attempt was made to carry out the training and the tests during the morning hours, before the heat lowered the test subjects' motivation.

The relative humidity increased during spring 1969 and 1970 from 43 % (SD 3.1 %) during the first test period to 50 % (SD 3.5 %) during the last test period. During the first period of training the relative humidity was 42 % (SD 1.1 %) and during the second 47 % (SD 1.8 %). In the autumn of 1970 the relative humidity fell slightly from 45 to 41 %.

VIII RESULTS

1 The correlation between initial work capacity and measures of physical dimensions and activity

The initial work capacity varies with heart volume and to a certain extent with weight and height, as shown in Table 3 (It is a pure coincidence that initial work capacity also varies with the frequency of training, since the training programs were assigned to the test subjects by a drawing of lots.)

The initial work capacity is better explained by heart volume than by weight and height combined and is equally well explained by heart volume alone than by heart volume and weight or height combined.

If the initial work capacity is expressed per kg of body weight, one quite naturally obtains a lower relationship with weight, height and heart volume (Table 3). On the

TABLE 3 The covariation between initial work capacity and anthropometric measures and other data (simple and multiple correlation coefficients)

Variable	Absolute work capacity		Work capacity per kg body weight	
	CSW	CPW	CSW	CPW
Age	0.18	0.16	-0.12	-0.09
Height	0.36	0.28	-0.20	-0.20
Weight	0.50 ^{***}	0.45 [*]	-0.28	-0.21
Heart volume	0.69 ^{***}	0.76 ^{***}	0.15	0.31
Physical activity:				
occupational	0.09	0.21	0.34	0.46 [*]
recreational	0.32	0.20	0.64 ^{***}	0.46 [*]
oking	-0.23	-0.33	-0.25	-0.35
frequency	0.50 [*]	0.44	0.38	0.33
duration of session	-0.07	-0.18	0.25	0.30
intensity	0.30	0.20	0.09	0.02
Height ²	0.36	0.28	-0.20	-0.20
Body surface area	0.50 ^{**}	0.44 [*]	-0.26	-0.26
Height and weight	0.50 [*]	0.45	0.28	0.22
Heart volume and weight	0.69 ^{**}	0.77 ^{***}	0.58 ^{***}	0.70 ^{***}
Heart volume and occupational physical activity	0.69 ^{**}	0.77 ^{***}	0.36	0.53 [*]
Heart volume and recreational physical activity	0.79 ^{***}	0.81 ^{***}	0.67 ^{***}	0.58 ^{***}
Smoking and occupational and recreational physical activity	0.34	0.38	0.73 ^{***}	0.66 ^{**}
Heart volume, smoking, occupational and recreational physical activity	0.80 ^{**}	0.81 ^{***}	0.71 [*]	0.67 [*]

CSW = capacity for short work

CPW = capacity for prolonged work

TABLE 4 Work rate, heart rate at the end of work test, work capacity and the coefficient of regression of log work time on log work rate during the three test periods for the capacity for short-time work (CSW) and for prolonged work (CPW). Test period I = 4 and 2 days before training, test period II = during training, test period III = 2 and 4 days after training. Significance of the mean of individuals differences between various test periods are given in the last three columns.

Test period		I	I	II	III	I-II	I-III	II-III
<i>Capacity for short work (CSW)</i>								
Work rate	\bar{x} SD	1563	237	1638	238	1779	252	***
kpm x min ⁻¹	range	1050-2050		1280-2137		1350-2338		***
Heart rate	\bar{x} SD	191	9	191	9	190	9	
beats x min ⁻¹	range	168-204		172-206		170-204		
W _{max} 6 min	\bar{x} SD	1556	235	1714	275	1813	300	***
kpm x min ⁻¹	range	1107-2114		1294-2243		1375-2331		***
<i>Capacity for prolonged work (CPW)</i>								
Work rate	\bar{x} SD	991	172	1157	236	1287	247	***
kpm x min ⁻¹	range	650-1420		750-1650		900-1756		***
Heart rate	\bar{x} SD	179	13	175	13	181	11	**
beats x min ⁻¹	range	149-200		153-194		158-202		
W _{max} 90 min	\bar{x} SD	988	173	1156	213	1258	227	***
kpm x min ⁻¹	range	662-1365		781-1587		861-1662		***
Coefficient of regression	\bar{x} SD	-5.91	1.23	-7.07	1.34	-7.36	1.08	***
	range	-4.13/-8.88		-4.24/-10.44		-5.69/-9.52		

other hand, the test subject's occupational and recreational physical activity correlate better with initial work capacity expressed per kg of body weight than when taken in absolute terms.

2. The change in work capacity and its variation with the intensity frequency and duration of training

The work capacity after training differs significantly from the values obtained prior to training. There is no significant difference in the heart rate at the end of work (see Table 4).

2.1 Short time work capacity

The absolute change in (absolute) short time work capacity is primarily due to the intensity of training and consequently the total amount of work during training (see Table 5). The frequency of training has also a certain importance, while the covariation with the duration of training is not significant. (The correlations are the same if the change in work capacity is expressed per kg of body weight or as a percentage of the initial value, and therefore these variants are excluded in the following.) Following multiple regres-

TABLE 5 Covariation between change in work capacity and the frequency duration and intensity of training, initial work capacity and some other variables (simple and multiple coefficients of correlation)

Variable	Change in CSW			Change in CPW		
	absolute	per kg b wt.	%	absolute	per kg b. wt.	%
Training: frequency (actual)	0.36	0.31	0.23	0.42	0.37	0.24
duration of session	0.18	0.23	0.16	0.23	0.32	0.29
Intensity (actual)	0.60***	0.53**	0.55**	0.63***	0.55**	0.54**
Intensity as mean work rate: train/W _{max} 6 min	0.33	0.28	0.30	0.28	0.20	0.15
Intensity as HR _{work} /HR _{max}	0.32	0.25	0.31	0.28	0.18	0.19
Mean work rate during training	0.44*	0.34	0.30	0.43*	0.31	0.19
Total work during training	0.60***	0.58**	0.48*	0.58***	0.57**	0.41*
Initial CSW	0.31	0.19	0.04	0.39*	0.26	0.07
Initial CPW	0.22	0.13	0.02	0.11	-0.02	-0.24
Initial CSW per kg b. wt.	0.19	0.24	0.03	0.31	0.39*	0.11
Initial CPW per kg b. wt.	0.13	0.20	0.02	0.03	0.10	-0.23
Frequency and intensity of training	0.71**	0.63**	0.61**	0.78***	0.69***	0.60**
Frequency intensity and duration of training	0.74***	0.67**	0.63**	0.82***	0.76***	0.67**
Frequency intensity and duration of training and initial work capacity	0.74**	0.69*	0.70**	0.84***	0.81***	0.83***
Age	0.13	0.04	0.10	0.09	-0.03	0.05
Wt	-0.04	-0.20	-0.14	0.05	-0.15	-0.03
	0.17	-0.06	0.01	0.15	-0.12	-0.03
volume	0.28	0.13	0.10	0.17	-0.01	-0.10
Physical activity occupational	0.32	0.42*	0.31	0.14	0.22	0.05
Physical activity recreational	0.07	0.14	0.00	0.14	0.23	0.05
Smoking	0.17	0.15	0.25	0.19	0.15	0.32

CSW = capacity for short work

CPW = capacity for prolonged work

sion analysis the two most important factors of the training program—intensity and frequency—account for 51 % of the variance of the change of short time work capacity. It does not prove worthwhile to include duration or initial work capacity in the equation (see Table 6).

In Table 7 the increase in short time work capacity for the different training programs has been calculated according to equation 3 in Table 6, and in Table 8 the increase in short time work capacity as a function of various combinations of training programs and initial short time work capacity has been calculated according to equation 4 in Table 6.

TABLE 6 Multiple regression between change in work capacity and the intensity frequency and duration of training and initial work capacity

Eq no.	Regression equation	r or R	s _e
<i>Change in capacity for short work (CSW) =</i>			
1	$-61 + 3.99 I^{**}$	0.60 ^{**}	104
2	$-171 + 4.14 I^{***} + 32.7 F^{*}$	0.71 ^{***}	93
3	$-207 + 4.14 I^{***} + 32.4 F^{**} + 0.5 D$	0.74 ^{***}	91
4	$-156 + 4.38 I^{***} + 36.5 F^{*} + 0.5 D - 0.051 CSW_1$	0.74 ^{**}	93
<i>Change in capacity for prolonged work (CPW) =</i>			
5	$-74 + 4.34 I^{***}$	0.63 ^{***}	103
6	$-206 + 4.51 I^{***} + 39.2 F^{**}$	0.78 ^{***}	88
7	$-254 + 4.55 I^{***} + 38.8 F^{***} + 0.7 D$	0.82 ^{***}	80
8	$-121 + 4.92 I^{***} + 48.3 F^{**} + 0.6 D - 0.184 CPW_1$	0.84 ^{***}	76
I	= intensity as the work rate in per cent of the highest work rate the subject can sustain during the training session		
F	= no. of training sessions per week		
D	= duration of training session in min		
CSW ₁	= capacity for short time work (W _{max} 5 min) before training in $\text{kpm} \times \text{min}^{-1}$		
CPW ₁	= capacity for prolonged work (W _{max} 90 min) before training in $\text{kpm} \times \text{min}^{-1}$		
r	= linear coefficient of correlation		
R	= multiple linear coefficient of correlation		
s _e	= standard deviation of residuals round the regression line		

TABLE 7 Predicted absolute change in work capacity as a function of the training program in $\text{kpm} \times \text{min}^{-1}$ (within brackets change in per cent) Duration is expressed in min, frequency in times per week and intensity as the work rate in per cent of the highest work rate that can be sustained during the training session.

Duration	15			60			120		
Frequency	1	3	5	1	3	5	1	3	5
Intensity									
<i>Change in capacity for short work (CSW)</i>									
50	42 (3)	106 (7)	171 (11)	66 (4)	131 (8)	196 (13)	98 (6)	163 (11)	228 (13)
75	146 (9)	211 (14)	276 (18)	170 (11)	235 (15)	300 (19)	203 (13)	268 (17)	332 (21)
100	250 (16)	315 (20)	380 (24)	274 (18)	339 (22)	404 (26)	307 (20)	372 (24)	437 (28)
<i>Change in capacity for prolonged work (CPW)</i>									
50	23 (2)	100 (10)	178 (18)	55 (6)	133 (13)	210 (21)	98 (10)	175 (18)	253 (26)
75	137 (14)	214 (22)	292 (30)	169 (17)	246 (25)	324 (33)	212 (21)	284 (29)	367 (37)
100	250 (25)	328 (33)	406 (41)	283 (29)	360 (36)	438 (44)	325 (33)	403 (41)	481 (49)

TABLE 8. Predicted absolute change in the capacity for short time work (CSW) in $\text{kpm} \times \text{min}^{-1}$ as a function of the intensity (in %) and frequency (times per week) of training and initial CSW ($\text{kpm} \times \text{min}^{-1}$) when training 60 min per session (eq no 4 in Tabl 6) In order to calculate equivalent values when training 15 or 120 min per session subtract 23 or add 31 $\text{kpm} \times \text{min}^{-1}$ respectively to the given values.

Intensity ^a	50			75			100		
Frequency	1	3	5	1	3	5	1	3	5
Initial CSW									
1000	80	153	226	189	262	335	299	372	445
1100	75	148	221	184	257	330	294	367	440
1200	70	143	216	179	252	325	289	362	435
1300	65	138	211	174	247	320	284	357	430
1400	60	133	206	169	242	315	279	352	424
1500	55	128	201	164	237	310	274	347	419
1600	50	123	196	159	232	305	269	341	414
1700	45	118	190	154	227	300	263	336	409
1800	40	112	185	149	222	295	258	331	404
1900	34	107	180	144	217	290	253	326	399
2000	29	102	175	139	212	285	248	321	394
2100	24	97	170	134	207	280	243	316	389

^aIntensity as the work rate in per cent of the highest work rate that be sustained for 60 minutes.

2.2 Prolonged work capacity

Likewise, the absolute change in (absolute) prolonged work capacity is primarily a function of the intensity and frequency of training or the total amount of work during training (see Table 5). (As with short time work capacity the correlations are the same if the change in work capacity is expressed per kg of body weight or as a percentage of the initial value instead of absolute figures, and these variants are therefore excluded in the following.)

As was the case with the change in short time work capacity the change in prolonged work capacity proved, following multiple regression, to be primarily a function of the intensity and frequency with which the training was conducted (see Table 6). If in addition to intensity and frequency duration or initial work capacity were introduced as a third explaining variable, the coefficient of multiple correlation increased but little (from 0.78 to 0.82 and 0.82 respectively).

In Table 7 the increase in prolonged work capacity for the different training programs has been calculated according to equation 7 in Table 6 and in Table 9 the increase in prolonged work capacity as a function of various combinations of training programs and initial capacity for prolonged work has been calculated according to equation 8 in Table 6

TABLE 9 Predicted absolute change in the capacity for prolonged work (CPW) in $\text{kpm} \times \text{min}^{-1}$ as a function of the intensity (in %) and frequency (times per week) of training and initial CPW ($\text{kpm} \times \text{min}^{-1}$) when training 60 min per session (eq. no. 8 in Table 6). In order to calculate equivalent values when training 15 or 120 min per session subtract 26 or add 35 $\text{kpm} \times \text{min}^{-1}$ respectively to the given values.

Intensity ^a	50			75			100		
Frequency	1	3	5	1	3	5	1	3	5
Initial CPW									
600	97	194	291	220	317	414	343	440	536
700	79	176	272	202	299	395	325	421	518
800	61	157	254	184	280	377	306	403	500
900	42	139	235	165	262	358	288	385	481
1000	24	120	217	147	243	340	270	366	463
1100	5	102	198	128	225	321	251	348	444
1200	-13	83	180	110	206	303	233	329	426
1300	-32	65	161	91	188	284	214	311	407
1400	-50	46	143	73	169	266	196	292	389

^aIntensity as the work rate in per cent of the highest work rate that can be sustained for 60 minutes.

3 The relationship between the capacity for prolonged and short time work and the change in this relationship

The relationship between the capacity for prolonged and short time work was highly significant both prior to the training ($r = 0.89$) as well as after 11 weeks of training ($r = 0.95$). The difference in correlation is not significant.

In absolute terms, the average increase in the capacity for short time work was almost as great as the increase in the capacity for prolonged work (256 $\text{kpm} \times \text{min}^{-1}$ and 270 $\text{kpm} \times \text{min}^{-1}$ respectively). A comparison of the figures for the absolute increase in Table 7 shows that no training program had any selective influence on either prolonged or short time work capacity.

In as much as the capacity for short time work is greater than that for prolonged work, an equally great increase for each implies in absolute terms that the relationship between the capacity for prolonged and short time work is changed and also implies a change in slope for the relationship between the logarithms for work time and work rate. The coefficient of regression increased from an average of -5.9 before training to -7.1 after four weeks of training and to -7.4 after the completion of training. The difference in slope before and after training is highly significant (see Table 4).

IX. DISCUSSION

1 Methodological problems

1.1 The mode of expressing work capacity

In this study work capacity is expressed as the highest work rate the individual can sustain for 6 or 90 minutes, respectively. From a logical point of view this is not correct, since the maximum work time is a consequence of the work rate and not vice versa. The fact that this method of expression was nevertheless used is due, among other things to the following circumstances. If, after training, each test subject had performed at the work rates which prior to training resulted in work times of 6 and 90 minutes, the work time would in certain cases have been more than doubled or even doubled many times over. This was not desirable, since maximum work tasks of widely different duration do not have the same physiological significance. For this reason the work rates were chosen in such a way that the maximum work was approximately 6 or 90 minutes respectively. Furthermore, with the use of different work rates one cannot make a comparison between the individual's results before and after training (or between results for different individuals) solely on the basis of work time. However the mode of expressing work capacity introduced by Tornvall (1963) does make possible a comparison of results obtained from tests at different work rates, and it has therefore been used in the present study.

1.2 Conversion of maximum work time at a certain work rate into maximum work rate for a set work time

In individual cases the work time of course deviated from 6, or 90 minutes respectively even though the work rate was chosen with these work times in mind. Thus, the highest work rate the test subject should have been able to sustain for 6 or 90 minutes respectively must be calculated on the basis of actual work rate and work times. For this purpose, formula (1) and (3), respectively (see section VI.3) were used. The constant b in these formulas is the slope of the straight line, describing the relationship between the logarithmic values for work time and work rate.

In calculating the work capacity one should ideally use the 'true' value for the constant b . However this true value is unknown, which means that one has to use an approximate value. In the present study the average value for the constant b was -5.9 , -7.1 and -7.4 respectively during the first, second, and third test occasions, while the average regression in the two sets of military conscript material previously reported (Tornvall 1963, Ahlborg 1966) was -5.0 and -5.7 respectively. However during these previous studies the bicycle ergometer model AM 361 was used, i.e., the type with which the actual work rate increases during work of long duration (see Table 2). This probably means that the absolute value of the coefficient of regression was actually somewhat greater than 5.0 and 5.7.

However as long as the actual work times are close to the reference time (6 or 90 minutes), it hardly matters whether one sets the value of the constant b at -5.0 , -6.0 or -7.5 . This can easily be checked by a few calculations.

If as in the present study each test subject has been tested at two different work rates in each round of tests, then it is possible to make an individual estimate of the coefficient of regression. However this estimate is probably so uncertain that it is of no advantage to use it in calculating work capacity.

Comparisons have been made between work capacity calculated according to the following coefficients of regression: a) the estimate made on an individual basis at each round of tests; b) the average for the group at each round of tests (-5.9 , -7.1 and -7.4); c) -5.0 ; d) -6.0 . The results are approximately the same in all four cases (see Table 10), and one can summarize in the following way what has been learned from this comparison and observations made in other studies:

1. In bicycle ergometer tests of this type the work rate ought to be adjusted to the individual, in order that actual work time will be close to the reference time.
2. As a rule, in calculating work capacity one ought to use a mean value for the constant b . This mean value can be calculated on the basis of the test material at hand or equivalent material.
3. Even if the actual work time departs considerably from the reference time, it is doubtful whether it is appropriate to use individual values for the constant b in calculating work capacity if these values are based solely upon two tests. It is probably more appropriate to use the mean value for the constant b especially if the test material is relatively homogeneous.

The results presented in this study have all been calculated on the basis of the mean value obtained at each of the three rounds of tests.

TABLE 10. Difference between values of physical work capacity calculated for different b -values ($n = 3 \times 27$)

Employed b -value		$\bar{W}_{\max b \min}$		$\bar{W}_{\max 90 \min}$	
I	II	\bar{d}	SD_d	\bar{d}	SD_d
-5.0	-6.0	9	24	-3	13
-5.0	b_w	17	41	-6	22
-5.0	b_{ind}	16	42	-7	21
-6.0	b_w	7	18	-2	9
-6.0	b_{ind}	6	24	-4	12
b_w	b_{ind}	-1	20	-2	11

b_w = weighted mean of slope during each test period

b_{ind} = individual values of slope during each test period

1.3 Reliability in determining work capacity

The methods used in determining the capacity for short time and prolonged work have been proved to be highly reliable (Nordesjö 1974 b). During a test and retest procedure (without intervening training) it was shown that the result at the retest was on the average somewhat higher than the value obtained in the first instance. The average increase was 6 and 52 kpm \times min⁻¹ for short time and prolonged work capacity respectively (Whether this increase can be said to be due to habituation or to the fact that the first work test involved a certain training—see section IX.1.7—is of no significance in this context.) This means that one underestimates the precision of a single determination if one calculates it according to the commonly used formula.¹ For this reason, a variant of this formula has been used instead.² The precision of a single determination of short time and prolonged work capacity calculated in this way is 62 and 47 kpm \times min⁻¹ respectively.

1.4 Intended and actual intensity

According to the test plan, 9 test subjects were to train at 50 %, 9 at 75 %, and 9 at 100 % of the highest work rate the test subject could sustain for 15, 60, or 120 minutes—i.e., the average intensity was to be 75 %. In as much as the work capacity increased during training, the intensity diminished, relatively speaking. In order to be able to adjust the work rate during training so that the intensity was once again the intended, the test subject had to carry out a new short time and prolonged work test after four weeks, when the first half of the training was concluded. The average intensity was 78 and 77%, respectively at the beginning of the first and fifth week of training, but it was lower (68 and 72%, respectively) at the end of the fourth and eighth week and the average mean intensity was 73 and 74.5%, respectively. However the two work tests carried out after the fourth week cannot be disregarded from the standpoint of training but must be included as two training sessions during which the intensity was 100 %. The average mean intensity then turned out to be 75 %. Consequently the obtained intensity for the whole group was rather close to the intended in spite of considerable increases in work capacity in certain cases.

Ideally the work rate should be adjusted every day of training in parallel with improvement in the physical fitness of the test subject. However this is hardly possible with the method and the definition of intensity used in this study. If instead intensity is defined as a certain heart rate, then one can allow the heart rate to control the work rate during each training session so that the intensity—i.e., the heart rate—always remains the same. However there are certain disadvantages with this arrangement. The control of heart rate requires either an increased number of test personnel or a sophisticated electronic regulation system. Moreover it was found in the present study that the difference in heart rate between two training sessions, when training at the

$$1 \quad \sqrt{\frac{\sum d^2}{2n}}$$

$$2 \quad \sqrt{\frac{\sum (d-d)^2}{n(n-1)}}$$

same work rate and on two consecutive days of training varied between -25.5 and $+24.6$ beats \times min^{-1} ($\bar{x} = -0.3$ SD $= 7.3$ beats \times min^{-1}).

The sum of differences between the final heart rate during the first and last training session of both rounds of training can be taken as a measure of the decrease in intensity (Table 12). An appreciable decrease in heart rate was registered only for those who trained at 100% intensity.

1.5 Different ways of expressing intensity

In this study the intensity of training has been expressed as a percentage of the highest possible intensity that can be sustained for the duration of the training session. Thus, in absolute terms the intensity 100% denotes different work rates depending upon whether the training session is 15, 60, or 120 minutes long, but this also means that the test subject is exhausted at the end of the training session.

In certain other studies the intensity of training is expressed as the work rate in per cent of the lowest work rate requiring full utilization of the maximum oxygen uptake ($= \% \dot{V}_{O_{2\max}}$). (Work done at this work rate is usually designated as maximal and work done at lower work rates irrespective of the duration of work or the degree of exhaustion as submaximal (Lange Andersen *et al.* 1971). Work done at loads higher than the lowest load which results in maximal oxygen uptake has been designated as super maximal (Karlsson 1971).)

Defined in this way 100% intensity always denotes the same absolute work rate of training for a stated test subject, whatever the duration of the training session. On the other hand, the degree of exhaustion is extremely dependent upon the duration of the training session. This is also the case when the intensity is referred to as work rate in percent of \dot{W}_{\max} . The simplest way of measuring intensity is, however, to state the absolute work rate of training.

In a few studies (Karvonen *et al.* 1957 Roskamm *et al.* 1966 a and b Kilbom 1971 a, b and c) heart rate has been used as a measure of the intensity of training. Such studies have then reported the difference between (mean) heart rate during training and heart rate at rest, i.e. pulse increase ($PI = HR_{\text{work}} - HR_{\text{rest}}$), relative to the pulse increase capability ($PIC = HR_{\text{max}} - HR_{\text{rest}}$) i.e. PI/PIC .

Since it is rather difficult to obtain a reliable value of an individual's heart rate at rest, it might, at least in homogeneous groups, be better to exchange individual heart rate at rest for the group mean in the formula above or to express training intensity as heart rate during training *per se* (HR_{work}) or relative to the maximal pulse ($HR_{\text{work}}/HR_{\text{max}}$).

1.6 Room temperature

In four cases the room temperature during the tests prior to and after training was $20-21^{\circ}\text{C}$ and $25-26^{\circ}\text{C}$ respectively. The significance of this is difficult to determine.

Studies concerning the importance of temperature have generally dealt with considerably larger intervals of temperature than those reported here. In a few studies an

session is not significant (Table 5), and does not become significant even if one takes into consideration the intensity and frequency with which the training was conducted (Table 6).

As mentioned earlier the significance of duration has been partially obscured by the fact that the intensity of training has been calculated on the basis of the duration of the training session. If intensity is instead expressed as work rate in per cent of W_{max} 6 min then there does exist a significant partial correlation between the increase in work capacity and the duration of the training session (see Table 11). Moreover this partial correlation is of the same magnitude as the partial correlation between frequency and change in work capacity. The partial correlation between duration and increase in prolonged work capacity is greater than that between duration and increase in short time work capacity.

Summarizing, one can then assert that all three factors—intensity, frequency and duration—are of essential importance for the change in work capacity but that the relative importance in the analysis of regression or correlation is dependent upon the chosen measure of intensity.

4 Training and heart rate

Maximum heart rate in this study is defined as the heart rate recorded at the end of work during tests on the capacity for short time work. However this heart rate is 3.5 beats \times min⁻¹ lower than the final heart rate recorded during the graded work tests to exhaustion which were conducted prior to the first test on short time work capacity. This is in agreement with Tornvall (1963) who found a corresponding difference of 5.5 beats \times min⁻¹.

The training did not lead to any decrease in heart rate at the end of the short time test (Table 3). On the other hand, there was a decrease in heart rate of 9 beats per min when the first and last session of each training round were compared (see Table 12).

In this study an attempt has also been made to illustrate the effect of stating intensity in terms of heart rate during training. An expression of the heart rate during training

TABLE 12. Change in heart rate during the last minute of the training session. Sum of differences between the first and last session in each training period. Mean for the 3 subjects who trained 1, 3 and 5 times per week.

Intensity of training, %*	Duration of training session, min		
	15	60	120
50	+14	-9	-2
75	+5	-4	-11
100	-18	-19	-29

*Intensity as work rate in per cent of the highest work rate that can be sustained during the training session.

(HR_{work}) for each individual is obtained by adding together the integrated heart rates from all training sessions and from the two middle tests and then dividing by the total time. The training heart rate has then been set in relation to the maximum heart rate (HR_{max}) and the heart rate at rest (HR_{rest}) in order to obtain the four measures of intensity described previously (see section IX.1.5). These four measures of intensity based upon heart rate are highly intercorrelated and therefore only HR_{work}/HR_{max} , i.e. an expression of the intensity of training which is not based upon heart rate at rest, is compared with other variables.

There is a small and insignificant correlation between HR_{work}/HR_{max} and change in work capacity ($r_{CSW} = 0.32$ and $r_{CPW} = 0.28$). Following multiple regression between change in work capacity and the measure of intensity given above, the frequency and duration of training the partial correlation between change in work capacity and intensity is highly significant (see Table 11). The regression equations are for change in short time work capacity (ΔCSW) = $-651 + 9.00 I + 37.7 F + 1.82 D$ and for change in prolonged work capacity (ΔCPW) = $-677 + 9.11 I + 43.9 F + 2.00 D$ where $I = HR_{work}/HR_{max}$ in percent, $F =$ number of training sessions per week, and $D =$ duration of training session in minutes.

The HR_{work}/HR_{max} registered at different times during the training period has been graphed in Fig. 4. The curves represent the mean value for those training 1, 3 or 5 times a week at the same intensity (per cent of the highest possible work rate during a

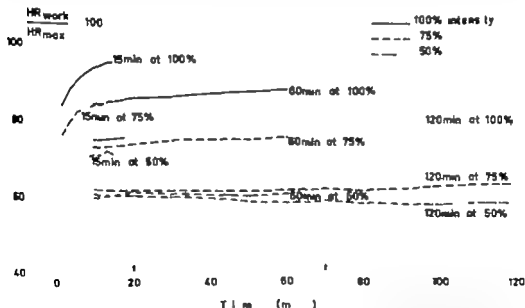


Fig. 4. Heart rate at set time during the training session (HR_{work} = sum of heart rates at set time of all training sessions/no. of training sessions) as percentage of the heart rate at end of test on short time work capacity (HR_{max} = mean of final heart rate at the first and last test of short time capacity). The curves represent a mean for those who trained 1, 3 or 5 times a week at the same intensity (% of the highest work rate that can be sustained during set time) and duration.

set time) and duration. Fig. 4 shows that certain training programs were the same with regard to HR_{work}/HR_{max} , namely

- 15 min x 75 % and 60 min x 100 %
- 60 min x 75 % and 120 min x 100 %
- 60 min x 50 % and 120 min x 75 %

Table 13 shows that the increase in the capacity for short time and prolonged work for equal values of HR_{work}/HR_{max} is greater when the duration of the training session is longer.

TABLE 13 Increase in physical work capacity as a result of training of varying duration but at approximately equivalent heart rates. Mean of the integrated heart rate after 10 min expressed as a percentage of maximal heart rate, work capacity expressed in $kpm \times min^{-1}$. Mean values for subjects who trained 1, 3 or 5 times/week.

Training program Intensity ^a	duration of session, min	Heart rate %	Change in CSW	CPW
75	15	84	213	182
100	60	86	340	460
75	60	74	308	256
100	120	77	489	428
50	60	60	134	163
75	120	62	229	248

CSW = capacity for short work

CPW = capacity for prolonged work

^aintensity as the work rate in per cent of the highest work rate that can be sustained during the training session.

5 The relationship between increases in the capacity for short and prolonged work

The absolute increase in the capacity for short time and prolonged work is of the same magnitude (on the average 256 respectively 270 $kpm \times min^{-1}$). The fact that the capacity for short time work is numerically greater than that for prolonged work means that the relative increases for both are of different magnitudes (16.5 and 27.4 % respectively) and that the coefficient of regression in the relationship log time-log work rate increases numerically from -5.9 to -7.4.

The covariation between the increase in the capacity for short time and prolonged work is highly significant ($r = 0.79$). Thus, none of the training programs have selectively affected the capacity for short time and prolonged work.

Normally the capacity for short time and prolonged work covariate with each other and both are intimately connected with body dimensions and maximum oxygen uptake (Tornvall 1963, Nordesjö 1974 a). Under normal conditions training can only give

marginal effects. In this context it is of little interest that one can vary the glycogen content of the muscles by means of prolonged work in combination with different diets and thereby selectively influence the capacity for prolonged work (Åhlborg *et al.* 1967 Hermansen *et al.* 1967).

6 Prediction of work capacity

As shown by Table 5 it is possible with a certain degree of certainty to predict the capacity for short time and prolonged work, even on the basis of such simple measurements as information on smoking habits and physical activity.

However an estimation of work capacity on the basis of the best combination of measurements for body dimensions and information on activities—i.e., heart volume and recreational physical activity—has a mean error (s) of 150 and 107 kpm \times min⁻¹ respectively for short time and prolonged work capacity.

Thus, it is only by conducting a work test that one can reduce the error of estimation to a reasonable magnitude, i.e., about 50 kpm \times min⁻¹.

7 The relationship between the amount of training and the effect of training

Siegel *et al.* (1970) proposed that the relationship between the amount of training and the increase in maximal oxygen uptake is S-shaped. In other words, there is practically no improvement in work capacity if the amount of training falls below a certain threshold. Furthermore, the increase in work capacity rapidly diminishes when the amount of training exceeds a certain limit. This hypothesis was based in part upon certain training studies (Karvonen *et al.* 1957 Hollman and Venrath 1963 Roskamm 1967) in which it was found that work capacity increased only when the increase in heart rate during training exceeded 60 % of the pulse increase capability ($HR_{max} - HR_{rest}$) i.e. about 130 beats \times min⁻¹. Furthermore, Mann *et al.* (1969) found that those test subjects who trained during 65 to 103 of projected 130 training sessions produced results of the same magnitude as those who trained 104 to 130 times.

The results obtained by Davies and Knibbs (1971) might also be explained by the existence of a threshold level, in as much as the test subjects in their study who trained at 50 % or less of $\dot{V}O_{2max}$ did not increase their maximum oxygen uptake.

On the other hand, Shephard (1968) has registered an increase of estimated maximum oxygen uptake at as low a training intensity as 39 % of estimated $\dot{V}O_{2max}$. Kilbom (1971 c) obtained training effects for women in the age group 19–48 years even when intensity was as low as 44 % of $\dot{V}O_{2max}$.

If according to principles referred to previously the training intensities used in the present study are converted into percentage figures of estimated $\dot{V}O_{2max}$ it will be seen that they correspond to 36–94 % of estimated $\dot{V}O_{2max}$. Thus, the lowest intensities are

then close to the 35 % level of $V_{O_{2max}}$ which an individual can sustain during an eight hour workday (including lunch and breaks) without becoming appreciably tired (Micheal *et al.* 1961 Åstrand, I. 1967 Bonjer 1968), a level which probably would not have any training effect. In spite of this, the three test subjects who trained at 50 % of the possible intensity in 120 minutes i.e. at 36–37 % of estimated $V_{O_{2max}}$ (41.4 % of pulse increase capability) achieved an increase of 88–157 $kpm \times min^{-1}$ (7–12 %) in the capacity for short time work ($W_{max 6 min}$) and an increase of 117–302 $kpm \times min^{-1}$ (12–41 %) in the capacity for prolonged work ($W_{max 90 min}$).

The contradictory reports on the existence of a threshold might possibly be explained by the fact that one seldom has complete and wholly reliable information on the activity patterns of the test subjects before and during the training studies. This fact leaves open the possibility that the test subject "compensates" for the programmed training by reducing his normal physical activity. If the test subject takes advantage of this possibility the result can give the impression that the threshold is higher than it actually is or that there is a threshold even though a threshold does not really exist. We do not have unobjectionable proof for the existence of a threshold. Until such is found perhaps the following, alternative hypothesis will serve just as well as a foundation for discussion and continued research: all increase of physical activity normally leads to an improvement in the capacity for physical work, provided that the physical activity does not already lie at a very high level.

Even if one can challenge the first part of Siegel's hypothesis in which he assumes the existence of a threshold, there is still much which bespeaks that his assumption of rapidly diminishing returns, once the training has exceeded a critical level, is correct. This would imply that, even if there does not exist any threshold, the effect of training is not a linear function of the intensity, frequency and duration of training.

Despite the fact that the data in the present study have been processed by means of linear regression analysis, most of the variation regarding the increase in the capacity for short time and prolonged work could be explained by the frequency, duration, and intensity of the training ($R = 0.74$ and 0.83 respectively). In order to check the possibility that a non-linear analysis might better explain the observed training effects, the residual variance for the increase in prolonged work capacity has been calculated on the basis of an approximative method supplied by Ezekiel and Fox (1959). The residual variance obtained in this manner does not differ significantly from that obtained by linear analysis. This in no way disproves Siegel's hypothesis. The present study was not designed to test this hypothesis. However the reported equations should not be used for extrapolations outside of the observed range.

8. Comparison between the present study the study by Shephard, and the study by Davies and Knibbs

Table 14 summarizes the test subjects' characteristics, experimental procedure and the result of the three studies. As a means of expediting comparisons between the three

TABLE 14 Summary of three studies wherein intensity frequency and duration of training have been varied in a systematic manner

	Shepard (1968)	Davies & Knibbs (1971)	Nordesjö (1974)
Subjects	non-athletic students	students of physical education	non-athletic students
Sex	not mentioned	males	males
Age	19-41	18-38	20-26
Initial $\dot{V}O_2$ max, lit x min ⁻¹ ml x kg ⁻¹ x min ⁻¹	3.30 43 (SD 5.8) ^a	3.35 48 (SD 4.4)	3.34 50 (SD 5.7) ^b
Training			
Type	treadmill	bicycle ergometer	bicycle ergometer
Intensity % of $\dot{V}O_2$ max	39.75 and 96 ^c	30.50 and 80	36-94 ^d
Frequency times per week	1.3 and 5	1.3 and 5	1.3 and 5
Duration of each session, min	5.10 and 20	5.10 and 20	15.60 and 120
Duration of training, weeks	3.4 and 6 ^e	8	8
Result			
Estimated mean increase of $\dot{V}O_2$ max, lit x min ⁻¹ ml x kg ⁻¹ x min ⁻¹	0.29 (9%) ^f	0.37 (<1%)	0.43 (13%)
Explaining variables in multiple regression r analysis of covariance and their significance			
Initial $\dot{V}O_2$ max	***	not included	not included
Intensity % of $\dot{V}O_2$ max		**	**
Frequency	*		
Duration			*

^a Estimated from submaximal work test (step test)

^b Estimated from maximal work test (\dot{W}_{max} 6 min)

^c In Shepherd's study work rate was not adapted to the individual's maximal oxygen uptake. On the contrary three fixed combinations of treadmill speed and slope were used which required 39.75 and 96 % of the subject's mean estimated $\dot{V}O_2$ max.

^d In the present study the intensity of training was 50.75 or 100% of the highest work rate the subject could sustain for set duration (15.60 or 120 min) and consequently the intensity varies between 36 and 94 % if it is expressed as per cent of $\dot{V}O_2$ max. If required the work rate was adjusted after 4 weeks of training.

^e The subjects who trained once a week trained for 6 weeks, those who trained 3 times a week trained 4 weeks and those who trained 5 times a week trained 3 weeks. No reasons for this are stated and the means of elucidating the importance of frequency are rendered more difficult by the described procedure.

^f Estimated increase in $\dot{V}O_2$ max if the subjects train 3 times a week at 75 % of $\dot{V}O_2$

^g Analysis of covariance

studies, the data in the present study has been converted into litres of oxygen uptake per minute. However it should be kept in mind that whichever of the three variables—intensity frequency and duration—best explains the increase in work capacity largely depends upon the extent to which they have been varied. There does not exist any common measure for the variation of intensity frequency and duration which would make it possible to make the variation of the three variables wholly comparable.

Shephard (1968) found that the absolute increase in estimated aerobic capacity was chiefly due to the individual's estimated, initial maximal oxygen uptake ($r = 0.59$). Following multiple regression a significant relationship ($p < 0.05$) could also be found between the increase in estimated aerobic capacity and the intensity and frequency of training.

The duration of the training session was, however not of significant importance. A certain training effect was obtained even when the test subject trained at low intensity (39% of estimated $\dot{V}O_{2\max}$), but the most effective training program was a combination of the highest intensity frequency and duration used in the study. The aerobic capacity of an individual with an initial oxygen uptake of $3.31 \text{ l} \times \text{min}^{-1}$ and weight of 76 kg can be calculated to increase $0.29 \text{ l} \times \text{min}^{-1}$ (9%), if he trains 3 times a week for 4 weeks at an intensity which equals 75% of estimated $\dot{V}O_{2\max}$, i.e. one of the middle programs.

Davies and Knibbs (1971) found that intensity was the most important factor for the increase of maximum oxygen uptake. Those who trained at 30 % or 50 % changed very little or not at all independent of the frequency and duration of training, but according to the figures for those who trained at 80 %, the extent of the change correlated both with the duration and frequency of the training. Even at the highest intensity (80 % of $\dot{V}O_{2\max}$) and greatest duration (20 min) the increase in aerobic capacity was very moderate from minus 1 to plus $9 \text{ ml} \times \text{kg}^{-1} \times \text{min}^{-1}$ (1–19 %). As a whole, the group's aerobic capacity increased by $0.37 \text{ ml} \times \text{kg}^{-1} \times \text{min}^{-1}$ (< 1 %).

In the present study the change in work capacity was found to be chiefly related to the intensity of training but also to its frequency and duration (see Table 14).

The most obvious difference in the results obtained by the three studies is the almost total lack of increase in aerobic capacity in the study of Davies and Knibbs. The test subjects in the last mentioned study were students of physical education, i.e., a group which probably is more physically active than the other two groups studied, even though the maximal oxygen uptake capacity is the same in the present study. Davies and Knibbs have also had the subjects exercising daily for 14 days immediately prior to the determination of the initial value for maximal oxygen uptake. Unfortunately further particulars of this pretraining have not been given. If such an extensive program of physical activity is not to influence work capacity this presupposes either that the subject is already rather well-trained or that the work rate is extremely low.

In the present study 2/3 of the training sessions were of very long duration (60 and 120 min), and as shown previously the duration of the training session is of significance (see section IX.3.4 and Table 11). But even the 9 subjects who trained for 15 minutes each session increased their estimated maximal oxygen uptake more than what was

reported in the other two studies where the training sessions were 5, 10 or 20 min long.

Moreover in the present study the work rates for training were adjusted after 4 weeks of training. This probably led to a greater increase in work capacity than that which would have resulted if the work rates had not been raised. But even after 4 weeks of training the average increase in estimated maximum oxygen uptake capacity was of the same magnitude ($0.27 \text{ l} \times \text{min}^{-1}$ 8 %) or greater than that reported in the other two studies. This is also the case for the groups which trained only 15 minutes each training session ($0.31 \text{ l} \times \text{min}^{-1}$ 9 %).

The number of training weeks in Shephard's study was not equal for those who trained 1, 3 or 5 times a week. As far as can be determined, Shephard has not taken this into consideration when processing his data. For this reason he may have under- or overestimated the importance of the frequency of training for the increase in work capacity.

In all three studies it appears as if the intensity of training is of greater importance for the increase in work capacity than the frequency and duration of training. However the importance of duration would need to be illustrated further by means of experiments in which the length of the training sessions varied over a greater interval of time, for example 5 to 120 minutes, but in which the frequency and intensity of the training was kept constant.

9 Regarding the suitability of expressing the increase of work capacity in percentage terms

When different individuals train according to the same training program, it usually happens that those who initially had high work capacity on an average achieve less of an increase in work capacity than those who initially had a low work capacity (Saltin *et al.* 1968). As previously pointed out (see section IX.3.1), this is probably more due to the fact that there is a greater number of well-trained individuals in the group with high work capacity as compared with the group with low work capacity than that initial work capacity *per se* is of any importance. The expression "the same training program" used above, implies that the intensities of the training are adjusted according to the individual and that, from a subjective point of view they are equal. When it comes to training work rates which, in an absolute sense, are equal, it is probable that both the initial degree of training and the initial absolute work capacity are of importance for the result.)

The suitability of expressing change in work capacity as a percentage of the initial value can be questioned, *inter alia* for the following reasons:

1. In the training of individuals with varying initial work capacity the increase is not directly proportional (but rather inversely proportional) to the initial work capacity.
2. Even if the absolute increase is equally great for all the test subjects, an obvious relationship is obtained between the relative increase and the initial level (on the premise that this varies).

- 3 Statements such as 'the work capacity increased by 10 per cent' are rather meaningless if one does not also state the measure used for work capacity as well as the initial level. It can hardly be said that all conceivable measures of work capacity increase percentually to the same extent during training. But once the increase is given in percentage terms, it is easy to be tempted into making statements which presuppose that the increase for different variables mentioned in various studies are directly comparable when expressed in percentage terms.

10 The coefficient of regression in the relationship between log work time and log work rate

10.1 Initial slope as a function of measures of physical dimensions and activity

The initial slope has weak but insignificant relationships with heart volume, smoking, occupational and recreational physical activity. The multiple correlation is 0.43 ($p > 0.05$) with heart volume, smoking, and occupational and physical activity as explaining variables and 0.46 ($p > 0.05$) with heart volume, smoking, and recreational physical activity as explaining variables. The partial correlations between initial slope and one of the other variables are not significant. The relationships are so weak that one must draw the following conclusion. It is impossible, on the basis of measurements of dimensions or activities, to determine in advance and with any great deal of certainty whether a person has a high or low capacity for prolonged work, relative to the capacity for short time work.

The result might, however, have been different if the coefficient of regression had been based upon more than two pairs of values.

10.2 Change in slope as a function of training

Training leads to a significant increase in slope, and there is a weak relationship ($R = 0.45$ $p > 0.05$) between the numerical increase in slope and the intensity, frequency and duration of training. Partial correlations are not significant. Thus, it was impossible to show the existence of any relationship between the change in the coefficient of regression and the length of the training sessions. Even though 1/3 of the training sessions were extremely long this did not lead to any selective increase in the capacity for prolonged work.

11 The test subjects' subjective experiences

In connection with the training, notations were made of the test subjects' experience of the undertaking. This can be summarized as follows:

- 1 In training at the 100 % level 5 times a week the test subjects had constant muscular ache, and their muscles were always tender and indurated. Although training at the

- 100 % level 3 times a week was experienced as strenuous, it did not cause constant muscular ache.
2. Training at the 50 % level for 60 or 120 minutes was felt to be too easy
 3. Training periods of 2 hours were too monotonous. Sessions of 15 and 60 minutes duration were more acceptable.

12. Choice of training program

A reasonable goal of training for the average 20 to 30 year old might possibly be an increase of about $250 \text{ kpm} \times \text{min}^{-1}$ in the capacity for short time and prolonged work. According to Table 7 an increase of $250 \text{ kpm} \times \text{min}^{-1}$ after two months of training can be achieved in several different ways, for example

- 1 time per week $\times 15 \text{ min} \times 100\%$
- 3 times per week $\times 60 \text{ min} \times 75\%$ and
- 5 times per week $\times 120 \text{ min} \times 50\%$

The test subjects' subjective experiences from the training indicate that, given a free choice among these three programs, they would prefer to train 3 times a week for 60 minutes at 75 % intensity. However, different individuals have different preferences. For example, some are unwilling to exert themselves (train at more than 50 %), while others enjoy exerting themselves almost to the point of utter exhaustion and do not consider the high training intensity as something negative. Then there are certain individuals who feel that they are more pressed for time than other people, etc. Thus, in spite of a common goal of training, different individuals can choose different ways of reaching this goal on the basis of varying preferences.

Figure 5 illustrates the effect of several different training programs on the capacity for short time work as based on the premise that the individual is initially rather untrained. The three continuous lines, which are based on equation 4 in Table II, show the effect of training 3 times a week for 60 minutes at relative intensity of 50, 75 and 100 % respectively. The increase is approximately the same, whatever the absolute value of the initial work capacity.

The dashed line represents the amount of increase in work capacity achieved by 197 military conscripts during the first part of their basic instruction, one which involves a relatively high degree of physical training (Linroth and Nordesjö 1968). The training was carried out in groups of about 30 persons, and within each group there was a rather wide range of initial physical fitness and work capacity. The relatively steep slope of this line is most likely due to the following conditions. When training is conducted on a group basis and the work capacity varies within each group, all of the members of the group will be training at approximately the same absolute intensity but at a varying amount of relative intensity. Those with the lowest degree of work capacity train at high relative intensity. Thus, they achieve a greater increase than those who have a high work capacity and who consequently train at a low relative intensity. Furthermore, individuals with high work capacity are on the average

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the mechanical efficiency of walking is 22 %. Even in this case the sharp slope of the curve can be explained with reference to varying relative intensity

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I INTRODUCTION

In recent years the effect of early undernutrition on the developing nervous system has attracted much attention. In particular the interest has been focused on the central nervous system and the possible causal relationship between undernutrition and the genesis of mental retardation.

As revealed by numerous animal experiments and a limited number of observations on humans, the most common forms of undernutrition i.e. calorie deficiencies and protein deficiencies (cf. McCance and Widdowson, 1968) may affect the physical development of the nervous system resulting in various kinds of deficits and distortions (Dobbing and Smart, 1973). These deficits and distortions have mostly been detected by nonhistological methods e.g. estimation of brain weight, determinations of the number of brain cells in tissue samples (Winick and Noble 1965 Winick, 1968 1969 Winick and Rosso, 1969) and determinations of myelin lipids, mainly cholesterol indicating the degree of myelination of cerebral white matter (Dobbing, 1964).

Platt and Stewart (1969) reported observations on spinal cords of young dogs showing that chromatolysis of the motor cells of the anterior horns and an increase in perineuronal oligodendrocytes are prominent but probably reversible phenomena caused by a low amount of protein in the diet.

The effect of early postnatal undernutrition on the growth and chemical maturation of single spinal cord motor neurons of the rat have been studied by quantitative cytochemical methods by Haltia (1970) and Haltia and Sourander (1970 a, b). In these studies it was shown that undernutrition during the first month of life markedly reduces the cytoplasmic RNA content and dry mass after lipid extraction. The reduced values both for RNA and dry mass, the latter corresponding to the bulk of proteins, indicate an impaired protein metabolism in the anterior horn motor neurons.

Hitherto only a very limited number of investigations have been published on the effects of protein calorie deficiencies on the developing peripheral nervous system (Clos and Legrand, 1970 Hedley Whyte and Mouser 1971 Hedley Whyte 1973). These authors have shown ultrastructurally that nutritional deprivation during early postnatal life causes a decreased number of myelin lamellae in the nerve fibres of the rat sciatic nerve. The two latter authors have also demonstrated that there is a decreased axonal circumference, indicating an impaired axonal growth.

Ever since Erlanger and Gasser (1937) made their fundamental discovery by using the cathode ray tube that nerve fibres conduct their impulses at different

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II MATERIAL AND METHODS

A. Animal material

1 Animals. Virgin rats of the Sprague Dawley strain 3 months of age were mated with males of varying ages up to 8 months. The time of fertilization was determined by vaginal smears twice daily. All litters used were adjusted to 8 pups at time of birth. Only male rats were used in this study. Control and undernourished rats ranging from 5 days to 180 days of age were investigated. As growth is no continuous process, the material had to be divided into several age-groups. Therefore control as well as undernourished rats were examined at 12 different ages. Each age-group consisted of at least 2 animals. Altogether 114 animals were investigated.

2 Animal environment. All control rats and those of the various experimental series were kept in plastic cages at a temperature of 25°C and in a relative humidity of 60 per cent. The room in which the animals were kept was illuminated from 6 a.m. to 6 p.m.

3 Diet. All animals were given the same pellets with a protein content of 18.8 cal. per cent concentrated fish protein (949A Astra-Ewos, Södertälje Sweden). The vitamin content in 10 gram pellets which was the amount consumed by an adult undernourished rat a day was above the daily requirement of an adult normal rat. The vitamin contents in 10 gram pellets in comparison with the normal requirement according to McCoy (1963) (within parenthesis) were: pyridoxin 50 µg (25 µg), niacin 250 µg (0), riboflavin 50 µg (40 µg) and thiamine 50 µg (10 µg). The amount of diet salt constituents in 10 gram pellets was also above the minimum requirement (within parenthesis) of normal adult rats according to McCoy (1963). Thus 10 gram pellets contained 4 mg (0.3 mg) magnesium, 49 µg (40 µg) zinc, 2 µg (0.4 µg) cobalt and 25.1 µg (5.3 µg) iodine.

4 Undernutrition. The method of undernutrition was that described by Chow and Lee (1964). This method implies pre- and postnatal undernutrition through a maternal diet restriction amounting to 50 per cent of the amount consumed by a control mother. Pregnant rats were given the restricted food amount from the first day of gestation. The restriction lasted during pregnancy throughout the suckling period until weaning. After weaning, the nutriment of the young rats was also reduced to 50 per cent of the amount consumed by age-matched control rats up to the age when they were sacrificed. For absolute body weights and bodily growth velocity estimated as the percental increment of normal adult value per one day see figures 1 and 3.

No evidence of increased sickness among undernourished rats was observed in the current experiment.

5 Nutritional rehabilitation. Eight undernourished litters were adjusted to 8 pups per litter at the time of birth and each litter rehabilitated separately from 0, 5 10 15 20 25 30 and 40 days of age respectively up to 90 days of age. Litters were rehabilitated from different ages of close intervals to cover up the time of accelerated fibre growth since this may coincide with a period of increased vulnerability (cf Dobbing, 1968 and Dobbing and Sands, 1971). The rehabilitation consisted of food *ad libitum* to the mother during the suckling period in litters where the rehabilitation was initiated before weaning. After weaning, the 8 litter-mates had free access to food up to 3 months of age when they were investigated.

Ventral and dorsal roots were investigated in rats from all rehabilitated litters, while the sciatic nerve was only investigated from the litter rehabilitated from 0 to 90 days of age. For body weights see figure 2.

B Histological procedure

1 Histological technique. The animals were anesthetized with ether. They were perfused with a combined paraformaldehyde-glutaraldehyde fixative containing 1.0 per cent paraformaldehyde and 1.25 per cent purified glutaraldehyde in 0.15 M cacodylate buffer of pH 7.15 with added CaCl_2 . The amount used was 1.5–2.0 ml/g body weight. This was followed by perfusion with the same volume of a solution four times the above mentioned concentrations. The nerves were then left *in situ* in the perfused animals for 4 hours, before they were dissected out. They were fixed in concentrated solution for another 12 hours then postfixed in 1.0 per cent OsO_4 for 2 hours, dehydrated and embedded in Epon 812 (cf Karnovsky 1965).

One micron thick sections were cut from a defined level of the sciatic nerve i.e. just proximal to the sciatic notch and from the spinal roots in the middle of their lengths, with a LKB-ultratome and stained with a combination of Mallory azur II and methylen-blue according to Richardson et al. (1960).

2 Methodological considerations. A prerequisite of necessity in studies of this kind is to eliminate errors in the histological procedures caused not only by the shrinkage due to fixation and embedding, but also by other artefacts some may be mentioned like beading effects and loss of fibres during histological treatment.

Previous reports on nerve fibre calibre spectra have generally been based on immersion fixed material. It is likely that the immersion fixation will cause errors reflected in the calibre spectrum since fibres centrally located in the nerve will be poorly preserved. (Luse 1960, Condie et al., 1961). This may be partly due to specific permeability properties acting in the perineurial sheaths (Shanthaveerappa, and Bourne 1963) which also might act as osmotic barriers during immersion fixation (Berthold 1968). Furthermore the manipulation with unfixed tissue during dissection preceding the immersion fixation may cause beading effects and fragmented or disrupted myelin sheaths (Ochs, 1963, 1965, Hildebrand and

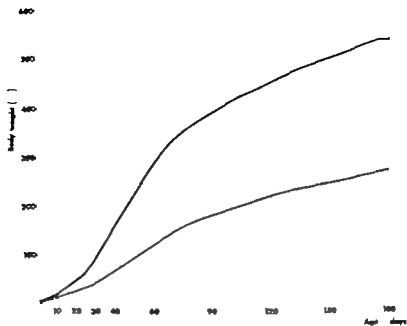


Fig. 1. Absolute body weight gain in control (—) and undernourished (---) rats.

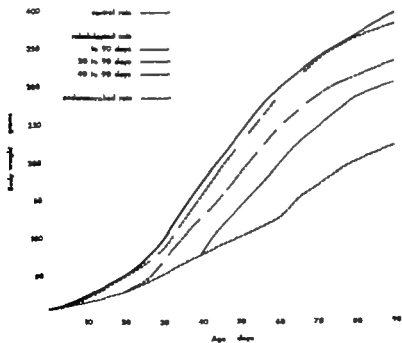


Fig. 2. Absolute body weight gain in rats nutritionally rehabilitated from different ages as compared with control and undernourished rats.

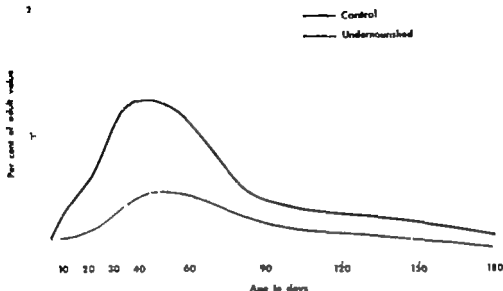


Fig. 3 Body weight increment in control (—) and undernourished (---) rats measured as percental weight gain per 1 day of normal adult value.

Skoglund 1971). The fixative itself is known to cause various degrees of shrinkage. Rexed (1944) showed in his study using formaldehyde that the concentration of the fixative is of extraordinary importance.

The recently usually used fixative in studies of calibre spectrum has been osmium tetroxide. According to Rexed (1944) this fixative causes no shrinkage, however Sherrington (1894) reported a 5 to 7 per cent shrinkage. According to Finean (1961) and Sjostrand (1963) using X-ray diffraction techniques, the shrinkage of the myelin period amounts to 10 per cent. Fixation with osmium tetroxide results in a good staining of large fibres whereas small fibres are indistinctly stained and therefore easily overlooked (Hildebrand and Skoglund, 1971 own observations, see figure 5).

Using perfusion-fixation the assumptions for a good preservation are naturally more favourable and mechanically induced artefacts by the dissection procedure can be fully avoided. Among perfusion fixatives generally used can be mentioned glutaraldehyde and a combination of paraformaldehyde and glutaraldehyde (Karnovsky 1965). According to Karnovsky the shrinkage caused by this latter combination is unusual despite its high osmolality.

The dehydration and embedding procedure may have caused the greatest artefacts in previous studies on calibre spectrum. Rexed (1944) found in his formaldehyde fixed material that the shrinkage due to dehydration and paraffin embedding was 30 per cent. Also paraffin embedded osmium tetroxide fixed peripheral nerves displayed a 20 per cent reduction in myelin sheath thickness (Williams and Wendel-Smith, 1960). The author's comparison between unfixed

per cent

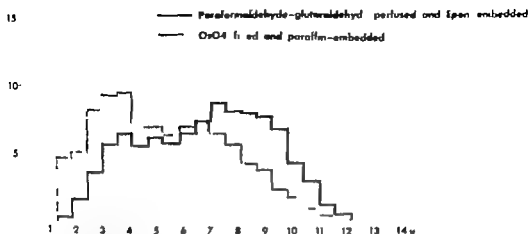


Fig. 4. Calibre spectra illustrating the difference in shrinkage between nerves treated according to the method used in this study (see under Histological technique) and OsO_4 -fixed and paraffin-embedded nerves.

frozen sections and paraffin sections of osmium fixed sciatic nerve showed a shrinkage in 90-day-old rats among the greatest fibres amounting from 14 to 19 per cent. Osmium fixed material embedded in araldite causes a reduction of the myelin period amounting to 30 per cent according to Finean (1961) and Sjöstrand (1963). A shrinkage of the same magnitude follows after glutaraldehyde fixation and Vestopal embedding (Karlsson 1966).

A drawback of considerable importance occurring in paraffin embedded nerves is the floating apart of nerve fascicles, loss of nerve fibres, and nerve fibres ending up obliquely to the long axis of the nerve, (Skoglund and Romero 1965 own observations see fig. 5) which inevitably will cause a somewhat distorted picture of the fibre distribution. By using perfusion fixation according to Karnovsky (1965) followed by Epon 812 embedding many disadvantages of earlier used methods can be avoided. This method will cause considerably less shrinkage than the earlier used osmium-paraffin method (figure 4).

Own comparisons between perfused and Epon 812 embedded sciatic nerve from one side and cryostat sections of unfixed sciatic nerve from the other side of the same animals showed among the thick fibres, a shrinkage less than 5 per cent in 90-day-old normal and undernourished rats. In this relatively small comparative material the shrinkage in undernourished rats was less than in normally nourished rats. In Epon 812 embedded nerves the loss of fibres were minimal as compared with paraffin embedded material, and fibres ending up obliquely were not observed (figure 5).

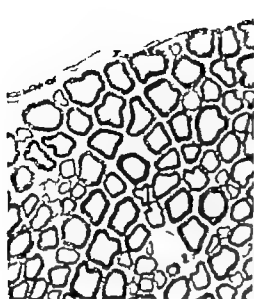


Fig. 5a.

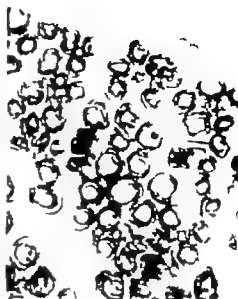


Fig. 5b

Fig. 5 Cross sections of sciatic nerves from 90-day-old control rats treated with different histological techniques, (a) according to the method used in this study (see under Histological technique) (b) OsO_4 -fixed and paraffin embedded nerve. Note in (b) fibres and axons obliquely to the long axis and indistinctly stained small fibres.

Consequently this method shows substantially improved assumptions for studies on calibre spectrum of peripheral nerves.

3 Fibre counting technique. Randomly chosen sectors corresponding to approximately one fourth of the cross section of the sciatic nerves and to approximately one half of the cross sections of the ventral and dorsal roots were photographed on Afga-IFF rollfilm in a Zeiss Photomicroscope. The films were enlarged to an overall magnification of 960 times. The entire diameters of the myelinated nerve fibres were measured with a particle size analyzer (Zeiss TGZ 3) according to the method of Romero and Skoglund (1965). This instrument consists of 48 electromagnetic counters which are connected over a resistance with an adjustable circular light spot. This light spot is projected on a glass screen on which the copies with the enlarged nerve fibres are placed. The light spot is adjusted to the size of the nerve fibre being measured. With a footswitch the current to the corresponding electromagnetic counter is closed and at the same time the current to a sum counter is closed. With this instrument some 500 to 800 fibres can be measured per hour.

For slight fibre irregularities measurement calculations were adopted. In a pear-shaped fibre the diameter was measured on its base. In a sea-star shaped fibre the largest diameter was measured, and if the fibre was oval shaped the minimum diameter was measured (cf Rexed 1944, Romero and Skoglund 1965). Only myelinated fibres were measured.

At the commencement of this study the number of myelinated nerve fibres of the sciatic nerve of age matched control and undernourished rats was compared. Preliminary results showed great individual variations in the number of nerve fibres both in control and undernourished rats. Therefore no conclusions could be drawn from this comparison. Since the number of rats never exceeded three in each group the material basis was considered much too small for an investigation of this kind, and this aspect was not studied further. From other species like the cat, great individual variations are known to occur in fibre number of a defined nerve in animals of the same age (Skoglund and Romero, 1965).

C. Fibre growth velocity

The fibre growth velocity of the total number of myelinated fibres has been estimated on the basis of the calibre spectra. Velocity curves were calculated for the sciatic nerve and the ventral and dorsal roots in control and undernourished rats. Growth velocity was expressed as the percental increment of the normal adult value extrapolated per one day.

D. Statistical methods and presentation of the material

The statistical calculation used for the comparison of the fibre distribution between undernourished and normal rats was test for trend in a contingency table with p-value determination by Edgeworth's expansion and a correction for continuity (Odén and Wedel, 1973). The comparison between the fibre distribution in undernourished sciatic nerves and spinal roots, and normal sciatic nerves and spinal roots was performed so that one single experimental nerve (sciatic or spinal root) was compared to all (2 to 3) nerves of the same type in normal age matched animals.

The presentation of the results is divided into two chapters dealing with the sciatic nerve and the spinal roots respectively. In each chapter the normal development will be described first, followed by the results illustrating nerve fibre development in undernourished and nutritionally rehabilitated rats.

The complete material is presented in tables I-IX, where the number of measured fibres in the different groups are given. To facilitate reading, material of special interest is presented in histograms with references in the text. In these histograms fibres of different diameters have been plotted in percentages of the number of fibres measured against a scale of even microns.

III RESULTS

A. Sciatic nerve

1. Calibre spectrum during postnatal development in control rats. The complete results obtained from the normal development of the sciatic nerve are summarized in table Ia and Ib

As seen from figure 6a, 5-day-old rats showed a fibre distribution which ranged from 0.5 micron to about 4 micron with the fibres concentrated between 1.5 and 2 micron. At the age of 10 days the largest fibres had reached a diameter of 5 micron and the peak had shifted to reach 2.2 micron. In 15-day-old rats a bimodal configuration had developed, one peak for thin fibres at 1.8 micron and one for thick fibres at 2.9 micron (figure 7a). After this age a pronounced and continuous increase in thick fibre calibre growth took place up to the age of 30 days. At this age the largest fibres had reached a fibre diameter amounting to 7.5 micron and three peaks had developed, one for thin fibres at 2.2, one for intermediate fibres at 4.1 and one for thick fibres at 5 micron (figure 8a). Between 30 and 40 days the largest fibres increased considerably in diameter and reached at the age of 40 days a diameter of almost 10 micron while the maxima did not change between these ages. From here on a moderate and continuous fibre diameter increase took place up to the age of 90 days, when the largest fibres had reached a diameter of 13 micron and the three peaks had reached 3.9, 5.0 and 7.3 micron (figure 9a). Between the ages 90 and 180 days the maxima of the thick fibres as well as of the intermediate fibres continued to show a slow increase in fibre diameters, whereas the maximum of thin fibres remained around 3.9 micron. At the age of 6 months the thick fibres were concentrated around 8.5 micron and the intermediate around 5.6 micron (figure 10a).

2. Calibre spectrum during postnatal development in undernourished rats. A tabulation of the complete results of the fibre growth development in undernourished rats is presented in table IIa and IIb

In the youngest group 5-day-old rats, the fibres of the sciatic nerve were distributed between 0.5 and almost 4 micron with a concentration of fibres around 1.8 micron (figure 6b). At the age of 10 days the fibres were distributed between almost the same diameters as in 5-day-old rats with the maximum still gathered around 1.8 micron. In 15-day-old rats, the fibres ranged between 1 and 6 micron with one peak at 2.2 micron. Between the ages of 20 and 30 days a continuous and rapid increase in fibre diameters took place and in 30-day-old undernourished rats the largest fibres had reached a diameter of almost 8 micron. Furthermore at this age the calibre distribution showed a bimodal configuration with one maximum for thin



Fig. 6a.



Fig. 6b

Fig. 6. Histograms of sciatic nerves of 5-day-old rats, (a) control rats, (b) undernourished rats. (For further explanation see text.)

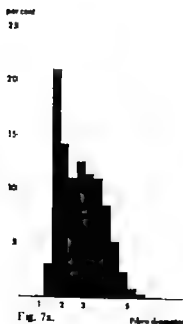


Fig. 7a.

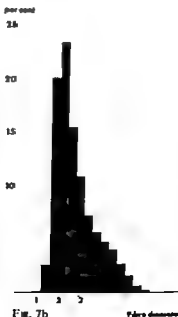


Fig. 7b

Fig. 7. Histograms of sciatic nerves of 15-day-old rats; (a) control rats, (b) undernourished rats. (For further explanation see text.)

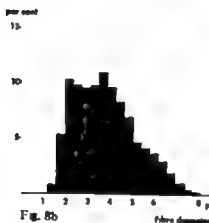
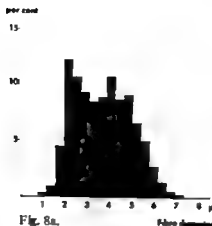


Fig. 8. Histograms of sciatic nerves of 30-day-old rats, (a) control rats, (b) undemourished rats. (For further explanation see text.)

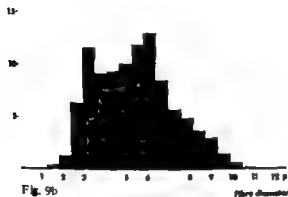
fibres at 2.2 micron and for thick fibres at 3.7 micron (figure 8b). In 40-day-old undemourished rats the maxima were unaltered as compared with 30-day-old rats, whereas the largest fibres had increased to a diameter of 9.5 micron.

Between the ages 40 and 90 days the fibre diameters showed a moderate and uniform increase and the maxima were shifted to reach 3.3 and 6.2 micron respectively in 90-day-old rats. The largest fibres were 11 micron thick at this age (figure 9b). From 90- to 180-day-old experimental rats the fibre diameter increase was slow but still at the age of 6 months the thick fibres showed a tendency to increase in fibre thickness, whereas the thin fibres were still concentrated around 3.3 micron. The thick fibres were gathered around 6.8 micron (figure 10b).

per cent



per cent



per cent



Fig. 9 Histograms of sciatic nerves of 90-day-old rats, (a) control rats, (b) undernourished rats, (c) rats rehabilitated from 0 to 90 days. (For further explanation see text.)

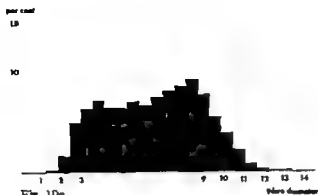


Fig. 10a



Fig. 10b

Fig. 10 Histograms sciatic nerves of 180-day-old rats (a) control rats, (b) undernourished rats. (For further explanation see text.)

3 Effect of postnatal nutritional rehabilitation on calibre spectrum. Three rats rehabilitated from birth to 90 days of age showed a calibre spectrum ranging from 1.5 to 11 micron thick fibres. The thick fibres were concentrated around 6 micron and the thin fibres showed a maximum at 3.7 micron. An intermediate peak had reached 5 micron (figure 9c). The complete results of fibre measurements of these rehabilitated rats are presented in table III

4 Comparison of calibre spectrum of control, undernourished, and rehabilitated rats. Comparing the development of sciatic nerve fibre growth of undernourished rats with that of control rats an impaired growth in thickness can be seen of both thin and thick fibres (figure 11a). The very largest fibres were also generally smaller in undernourished rats, however this difference was not that constant when comparing individual rats, as was the difference in fibre diameters of the maxima which occurred very constantly

The effect of early rehabilitation revealed a partial restitution of the calibre spectrum as compared with that of control and undernourished rats. Compare figures 9a, b and c.

The statistical evaluation of the differences between the fibre distribution of the sciatic nerve in undernourished and control animals showed highly significant values in all calculated groups, i.e. 5- 90- and 180-day-old rats ($p < 0.001$). The trend of the significant differences was systematic throughout the calculated groups, indicating a constant difference between the individual rats of the two groups.

Comparing the fibre growth velocity of the sciatic nerve in control and undernourished rats (see figure 11b) a difference in the appearance of the phase of most accelerated growth can be noted. The peak of this phase occurred for the control rats at the age of 10 days, for undernourished rats at the age of 25 days. The growth achievement of undernourished rats represented by the area below the dotted line in figure 11b was decreased as compared with that of control rats.

5. Observations on axonal diameter in control and undernourished rats. In addition to the measurements of the total fibre diameter the axonal diameters of sciatic nerve fibres were compared in two age groups of control and undernourished rats, namely in 30- and 150-day-old rats. The calibre spectrum of axonal diameters of 30-day-old undernourished rats exhibited lesser diameters than that of control rats of the same age. Thus in experimental rats 61 per cent of all myelinated axons were less than 2 micron thick, whereas the corresponding figure for control rats was 53 per cent. Even in 150-day-old rats the axonal calibre spectrum of undernourished rats was shifted to lesser diameters as compared with that of control rats (figure 12).

B Spinal roots

1 Calibre spectrum of ventral root (LV) during postnatal development in control rats. The complete results of fibre measurements of the ventral root in control rats are presented in table IVa and IVb.

In 5-day-old control rats the fibres of the fifth lumbar ventral root were distributed between 1 and 5 micron with one maximum at about 2 micron, contributed by 32 per cent of all myelinated fibres (figure 13a). At the age of 10 days two maxima had developed, one representing thin fibres at 1.8 micron and one constituted by thick fibres at 3.3 micron. The largest fibres were 5 micron thick (figure 14a). From here on to 20 days of age a very rapid and uniform increase in fibre diameters took place. In 20-day-old rats the largest fibres had shifted to reach 8 micron. The two peaks were made up of fibres 2.6 and 6 micron thick. Between 20- and 25-day-old rats the increase in fibre diameter slowed down and showed no increase between 30 and 40 days of age.

Between 40- and 150-day-old control rats, the thick fibres showed a moderate increase and reached a maximum of 9 micron at 90 days (figure 15a). At 5 months of age the peak for thick fibres had reached 11 micron. The thin fibres increased slowly from 40 up to 60 days and constituted at this age a maximum at 3.3 micron.



Fig. 11a.

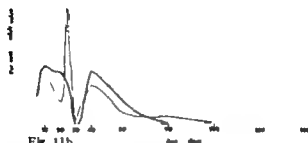


Fig. 11b

Fig. 11a. Increase in size of fibres comprising the peak of thick fibres of the sciatic nerve of control (—) and undernourished (---) rats.

Fig. 11b. Growth velocity curves of fibre thickness growth of total number of myelinated fibres in sciatic nerves of control (—) and undernourished (---) rats (For further explanation see text.)

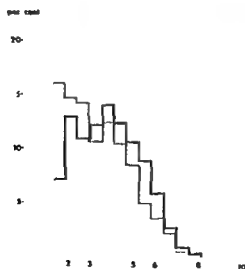


Fig. 12. Histogram illustrating the axonal diameters of sciatic nerve in control (—) and undernourished (---) rats 150 days old.

After the age of 2 months the thin fibres did not show any increase until the sixth month, when these had shifted to reach a maximum around 3.9 micron. The largest fibres had reached 16 micron and the maximum of thick fibres consisted of fibres 12 micron thick (figure 17a).

2. *Calibre spectrum of dorsal root (LV) during postnatal development in control rats.* A tabulation of the complete fibre measurements in dorsal roots of control rats is presented in table Va and Vb.

Five-day-old rats showed a fibre distribution between 0.5 and 4 micron with one peak at 2.2 micron (figure 13b). In 10-day-old rats the nerve fibres of the dorsal root were distributed in the same way (figure 14b). Between the ages 10 and 15 days the fibre diameters increased rapidly. The largest fibres had almost reached 8 micron in 20-day-old control rats. At this age the calibre spectrum showed a bimodal configuration with one peak for thin fibres around 2 micron and one for thick fibres at 4.1 micron. After 20 days of age the increase in fibre diameter declined and between 30 and 40 days no increase of thick fibres took place except for the very largest fibres which had shifted to 9 micron. At 40 days a third intermediate peak had developed at 3 micron. From this age up to 180-day-old rats a slow and continuous increase could be noted. Hence at the age of 90 days the fibres were distributed between 1.5 and 12 micron with the peaks at 2.7, 5.5 and 7.3 micron (figure 16a). Still at the age of 6 months both thin and thick nerve fibres showed a tendency of increase. In the oldest rats investigated the maxima had reached 3.3, 6.7 and 8.4 micron. The largest fibres had shifted to reach 14.5 micron (figure 17c).

3. *Calibre spectrum of ventral root (LV) in undernourished rats during postnatal development.* A tabulation of the complete results of the fibre measurements of the ventral roots in undernourished rats is presented in table VIa and VIb.

In the youngest group of undernourished rats investigated 5 days of age the fibres of the ventral root ranged from 0.5 to 4 micron with one peak at 1.8 micron formed by 44 per cent of all fibres (figure 13c). In 10-day-old rats the fibres were distributed between the same diameters but showed two peaks, one at 1.8 and one at 2.6 micron (figure 14c). In undernourished rats 15 days of age the ventral root fibres were distributed between 1 and 6.5 micron. The maximum for thin fibres was still gained by fibres 1.8 micron thick, while the maximum for thick fibres had shifted to 3.3 micron. Between the ages 15 and 25 days the thick fibres showed a rapid and uniform increase and had reached a peak at 6 micron at the end of this period. The peak for thin fibres was contributed by fibres 1.8 micron thick. Between 25 and 40 days of age no increase in growth of thick fibres took place. However, at 40 days the thin fibres had shifted to reach a peak at 2.2 micron. From 40 days onwards the thick fibres showed a relatively slow but continuous increase in fibre growth. This was also true for the thin fibres. Accordingly at the age of 90 days the maxima had reached 3.3 and 7.9 micron respectively (figure 16b). At 180-day-old undernourished rats the peaks were located at 3.9 and 11.4 micron for thin and thick fibres respectively. The largest fibres had now reached 14.5 micron (figure 17b).



Fig. 13a.

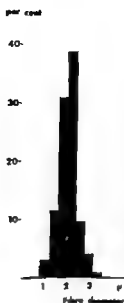


Fig. 13b



Fig 13c.

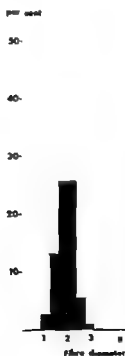


Fig. 13d

Fig. 13 Histograms of ventral and dorsal roots (LV) of control and undernourished rats 5 days of age: (a) ventral root of control rats, (b) dorsal root of control rats, (c) ventral root of undernourished rats, (d) dorsal root of undernourished rats. (For further explanation see text)

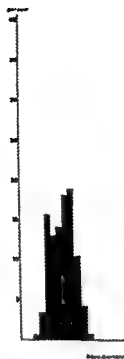


Fig. 14a



Fig. 14b



Fig. 14c



Fig. 14d

Fig. 14 Histograms of ventral and dorsal roots (LV) of control and undernourished rats 10 days of age (—) ventral root of control rats, (---) dorsal root of control rats, (·) ventral root of undernourished rats, (·) dorsal root of undernourished rats (For further explanation see text.)

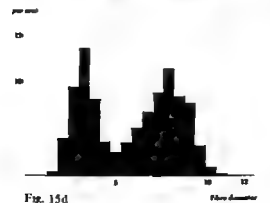
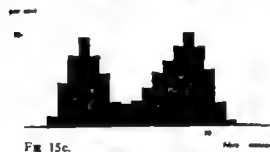
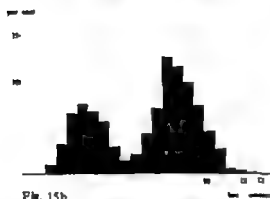


Fig. 15 Fibre distribution of ventral roots (LV) in 90-day-old rats, (a) control rats, (b) undernourished rats, (c) rat nutritionally rehabilitated from 5 to 90 days, (d) rats nutritionally rehabilitated from 40 to 90 days. (For further explanation see text.)

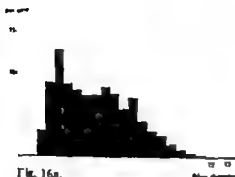


Fig. 16a.

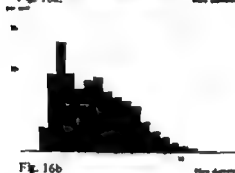


Fig. 16b

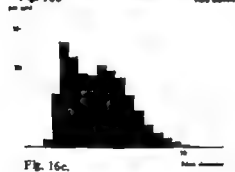


Fig. 16c.



Fig. 16d.

Fig. 16 Fibre distribution of dorsal roots (L.V) in 90-day-old rats, () control rats, (b) undernourished rats, () rats nutritionally rehabilitated from 5 to 90 days, (d) rats nutritionally rehabilitated from 40 to 90 days. (For further explanation see text.)

4 Calibre spectrum of dorsal root (LV) in undernourished rats during postnatal development. The complete material is presented in table VIIa and VIIb

In 5-day-old rats the fibres of the dorsal root were spread between 0.5 and 3.5 micron with one maximum at 1.8 micron (figure 13d). In 10-day-old rats the largest fibres had reached a total diameter of 4 micron but still only one maximum was visible at 1.8 micron (figure 14d). At the age of 20 days the maximum had first shifted to 2.2 micron and at this age the largest fibres were 6.5 micron thick. At the age of 25 days two peaks appeared for the first time, one for thin fibres at 1.8 micron and one for thick fibres at 4 micron. Between 20 and 25 days the thick fibres showed a rapid increase. At the end of the first postnatal month the thick fibres had shifted to a maximum at 4.4 micron. Between 30 and 40 days the thick fibres of the dorsal root showed no increase (figure 19a) whereas the thin fibres showed a rapid increase in fibre diameter and reached at the end of this period 2.2 micron. From 40 days of age the undernourished rats showed a slow and relatively uniform increase in diameter of thick fibres. The thin fibres showed no increase between 40 and 60 days, and after this period the increase was slow. At the age of 90 days the peaks were located at 2.7 and 5 micron (figure 16b). At the end of the observation period, i.e. in 180-day old undernourished rats, the maxima had reached 3.3 and 6.1 micron. The largest fibres had reached a diameter of 12 micron (figure 17d).

5 Comparison of calibre spectrum of normal and undernourished rats.

Ventral roots. Comparing the fibre growth development of undernourished rats with that of normally nourished control rats, a deficit in fibre growth can be noted among thick fibres, whereas the deficit in fibre growth of thin fibres is small. The timing of the most rapid growth of the nerve fibres occurred at the age of 20 days, both in control and undernourished rats (figure 18b). However the growth-spurt in the undernourished rats occurred to a lesser extent (figure 18b). Statistical treatment of three age groups: 5-, 90- and 180-day-old rats showed highly significant differences ($p < 0.001$) between individual undernourished and control rats. The trend of the differences was systematic in the groups mentioned, indicating a constant difference between the individual rats of the two groups.

Dorsal roots. Comparing the fibre growth development of the dorsal roots of undernourished rats on one hand and control rats on the other, great differences are obvious between the thick fibres, whereas the thin fibres show no differences.

At the age of 180 days the thick fibres of undernourished rats have reached only some 70 per cent of the normal diameter (figure 19a). In the dorsal root the timing of the phase of accelerated growth was retarded by 15 days in undernourished rats, and occurred to a markedly lesser extent (figure 19b).

Statistical evaluation of the same age groups as those evaluated for the ventral root also showed, among the dorsal roots, highly significant differences ($p < 0.001$) between undernourished and control rats concerning the fibre distribution. The trend of the differences here was also systematic indicating a uniform difference between individual rats throughout the groups evaluated.

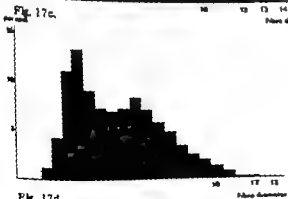
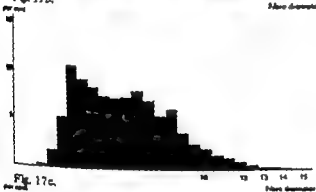
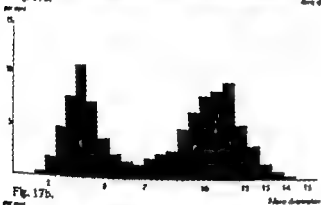
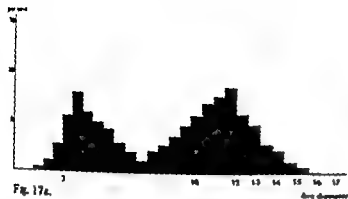


Fig 17 Fiber distribution of ventral and dorsal root (LV) in 180-d y-old rats, () ventral root of control rats, (b) ventral root of undernourished rats, () dorsal root of control rats, (d) dorsal root of undernourished rats (for further explanation see text)

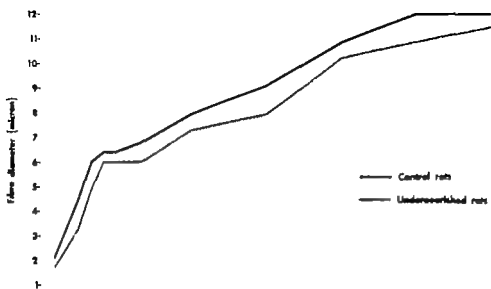


Fig. 18a

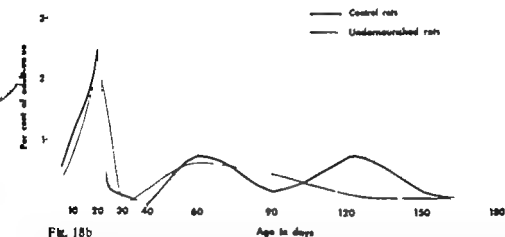


Fig. 18b

Fig. 18. Diagram (a) shows the increase in size of fibres comprising the peak for thick fibres of the ventral roots (LV) of control (—) and undernourished (---) rats. Diagram (b) shows the growth velocity curves of fibre thickness growth of the total number of myelinated fibres in ventral roots of control (—) and undernourished (---) rats. (For further explanation see text.)

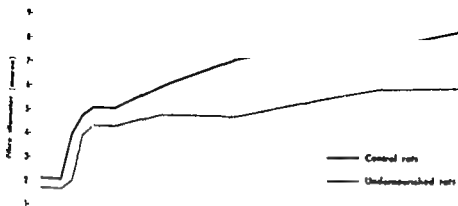


Fig 19a

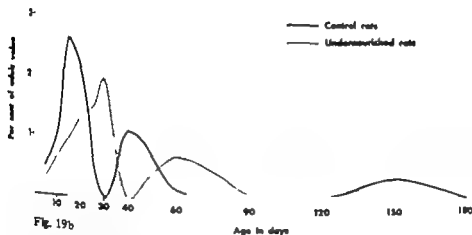


Fig. 19b

Fig. 19 Diagram (a) shows the increase in size of fibres comprising the peak for thick fibres of the dorsal roots (LV) of control (—) and undernourished (---) rats. Diagram (b) shows the growth velocity of fibre thickness growth of the total number of myelinated fibres in dorsal roots of control (—) and undernourished (---) rats. (For further explanation see text.)

6 *Effect of postnatal nutritional rehabilitation on calibre spectrum of ventral root (LV).* The complete results are given in table VIII

In all undernourished rats in which rehabilitation was initiated between 0 and 15 days of age (early rehabilitation) the calibre spectra of the ventral roots showed almost the same distribution of the fibres at the age of 90 days. Only minimal differences can be seen from the histograms of these rats. The fibres were distributed between 1 and 12–13 micron with two distinct peaks, one for thin fibres at 3.3 micron and one for thick fibres at 9 micron (figure 15c). In undernourished rats rehabilitated from 25–30 and 40 days of age up to 90 days (late rehabilitation) the calibre spectra ranged between 1 and 11–13 micron. The maxima for thin fibres were located at the same diameter as the corresponding maxima of undernourished rats subjected to early rehabilitation (i.e. from 0–5–10 and 15 days of age) whereas the maxima for thick fibres were located at 7.9 micron (figure 15d). In rats rehabilitated from 20 days to 90 days of age the maximum for thin fibres was located at 3.3 micron and the maximum for thick fibres at 8.4 micron.

7 *Effect of postnatal nutritional rehabilitation on calibre spectrum of dorsal root (LV).* The complete material is presented in table IX.

The calibre spectra of the dorsal roots of all undernourished rats subjected to rehabilitation were distributed between 1 and 10–11.5 micron. All animals of the entire groups of rehabilitated rats showed two maxima which were gathered around 2.7 micron for thin fibres and around 5 micron for thick fibres. Rats in which rehabilitation was initiated between 0 and 20 days of age (early rehabilitation), showed calibre spectra with a greater number of thin and intermediate sized fibres than the calibre spectra of rats rehabilitated from later ages (figure 16c). Conversely rats rehabilitated from 25–30 and 40 days of age up to 90 days of age (late rehabilitation) showed a greater number of thick fibres in their calibre spectra of the dorsal root than rats subjected to early rehabilitation (figure 16d).

8 *Comments. Ventral roots.* The calibre spectra of the ventral roots in rats subjected to early rehabilitation examined at the age of 90 days showed a fibre distribution similar to that of the ventral root in control rats of the same age (compare figures 15a and 15c) whereas rats subjected to late rehabilitation, examined at 90 days of age showed calibre spectra similar to that of the ventral root in equally old undernourished rats.

Using statistical comparisons between the fibre distribution of the ventral roots in rats rehabilitated between 5 and 90 days of age and that of control rats 90 days old, it was found out of 9 possible comparisons between individual rats of the two groups, 5 showed significant differences and 4 did not. However the trends of the significant as well as the nonsignificant differences were not systematic. This indicates that no differences in the fibre distributions between these two groups could be obtained. The opposite relationship was valid for the comparative conditions between the fibre distributions in the ventral roots of rehabilitated rats and undernourished rats, where the trend was systematic throughout these two groups. In this case with regard to fibre distribution the trend indicates that the

two groups were different and separate from each other (see table V). In addition it can be mentioned that statistical comparisons between the fibre distribution of individual rats belonging to the same control groups showed significant differences in some cases and not in some reflecting individual differences.

Dorsal roots. Comparing the fibre distribution of the dorsal roots of rats rehabilitated from different ages up to 90 days of age with control and undernourished rats of the same age the maxima were located at the same diameters as those of undernourished rats. In rats subjected to rehabilitation like in undernourished rats, at third maximum never developed. Rats subjected to early rehabilitation up to the age of 90 days, showed a slight increase in number of thin and intermediate sized fibres, whereas in rats subjected to late rehabilitation the free access to food was profited mainly by the thick fibres which increased in number when compared with those of undernourished rats.

Statistical comparisons between rats being rehabilitated from 5 to 90 days of age and undernourished rats 90 days old showed significant differences in their fibre distributions in 6 out of 9 cases. The trend between these two groups was not systematic which indicates a similarity between the two groups. The converse relationship was true with regard to the comparison between the fibre distribution in rats of the same rehabilitated group and that of control rats 90 days old. In this latter case the trend was systematic throughout all 9 possible comparisons. This indicates that these two groups are different as far as their fibre distribution of the dorsal root is concerned. For comparison see table X.

IV GENERAL DISCUSSION

The general pattern of the normal development of the calibre spectra of the sciatic nerve and its roots in the rat corresponds to that known from other species. Thus, the results obtained from the rat may be compared with previous results on cat (Skoglund and Romero 1965) and on man (Rexed, 1944) provided that corresponding developmental stages and not postnatal ages of the different species are considered.

The ventral root of the rat established a bimodal adult pattern of its calibre spectrum as early as at the age of 10 days with one peak for thin fibres and one peak for thick fibres. In the cat it has been shown that the thin fibres represent gamma-fibres (Eccles and Sherrington, 1930) innervating intrafusal muscle fibres in muscle spindles (Leksell 1945) and the thick fibres alpha-fibres innervating the main mass of extrafusal muscle. Compared to the ventral root, the dorsal root exhibited a slower development. The adult pattern of fibre distribution was reached at 40 days of age when three maxima were discerned, corresponding to I, II and III fibres according to the classification proposed by Lloyd (1943). Also the development of the calibre spectrum of the sciatic nerve was slow compared to the ventral root. At the proximal unbranched level investigated in this study a bimodal configuration with two distinct peaks was reached at the age of 15 days, while the adult pattern with three maxima was not established before the age of 30 days. The fibre types included in a mixed peripheral nerve such as the sciatic nerve are of course of heterogeneous origin and therefore no conclusions could be drawn concerning the relationship of the different maxima to defined fibre types.

Summarizing the development of the adult pattern of fibre distribution of the three nerves investigated it can be stated that the rat, like the cat (Skoglund and Romero 1965) showed the fastest development of the ventral root whereas both the dorsal root and the sciatic nerve showed a considerably slower development.

In man (Rexed, 1944) like in cat (Skoglund and Romero 1965) the increase in fibre size takes place during a relatively long span of life. In man the dorsal root of the first sacral segment still shows a tendency to increase in fibre thickness beyond the age of 25 years. As for the cat, even young adult animals still show an increasing fibre size. At the age of 180 days corresponding to one fourth of the average life expectancy of the rat, the thick fibres were thicker than in 150-day-old rats. It is thus obvious that the rat like the cat and the man displays an increase in fibre thickness proceeding into adult life.

A comparison of the normal development of the sciatic nerve in rat with that in pre- and postnatally undernourished rats revealed marked differences. In the sciatic

none of undernourished rats the calibre growth of all fibres seemed to be retarded but the retardation was more marked in the thick than in the thin fibres. Nerve fibres of intermediate size did not develop a well defined maximum in undernourished rats. When comparing the number of myelinated fibres in control and undernourished rats of the same ages, no significant difference was demonstrable. However no definite conclusions from such a comparison can be drawn since the number of rats examined in each age group was restricted to 3 animals, which was considered to be too small. It is known that considerable differences occur in fibre number between individual animals of the same age as shown by Stoglund and Romero (1965) for the cat and indicated for the rat by the author's observations.

The functional heterogeneity of the fibres forming the sciatic nerve and the differences noticed in the effect of undernutrition on thick and thin fibres demanded an investigation of the corresponding motor and sensory roots.

The postnatal development of spinal roots in undernourished rats was markedly retarded. This retardation was more pronounced in the dorsal than in the ventral root. In the dorsal root of 180-day-old undernourished rats the thick fibres showed a 30 per cent reduction in fibre thickness as compared with those of control rats. The intermediate sized nerve fibres did not develop a defined maximum in the dorsal root of undernourished rats. The location of the peak for thin fibres of the dorsal root was not affected by undernutrition.

In the ventral root, the thick fibres were markedly reduced in growth in thickness, whereas the thin fibres showed only a slight reduction in their calibre growth.

It is tempting to assume that the physiological consequence of the deficits found in nerve fibre calibres of peripheral nerves in undernourished rats will be manifested as reduced conduction velocities, since it is known that there exists a relationship between nerve fibre calibre and conduction velocity (Erlanger and Casper 1937 Hursh, 1939). In order to explore this possibility examinations are now in progress concerning the conduction velocity of peripheral nerves in undernourished rats (Jankowska and Sime).

In rats undernourished early in life, a reduced number of myelin lamellae of the sciatic nerve fibres has been ultrastructurally demonstrated by Chen and Legend (1970) and by Hedley Whyte and Meuser (1971). Chen and Legend found that for axons of about the same circumference the number of myelin lamellae was markedly reduced in undernourished rats. This observation was interpreted to indicate a direct effect of undernutrition on the process of myelination. However the results obtained by Hedley Whyte and Meuser showed that not only the myelination but also the circumferential growth of the axon is affected by early undernutrition. This is in agreement with the author's observations on the axonal diameter which appeared to be decreased in undernourished rats compared with age-matched control rats. The latter observations correspond well with the findings by Halata (1970) which showed a reduced protein and RNA content of the motor neurons of the anterior horn in undernourished rats, indicating an impaired process

metabolism. It may be assumed that an impaired protein metabolism will not only affect the growth of the perikaryon but also the growth of its axon. Furthermore the observed deficit in axonal growth in undernourished rats may in turn be responsible for an impaired myelination since it is known that the process of myelination is dependent on axonal protein flow and axonal growth (Friede 1972).

In order to find out whether the observed deficits in nerve fibre development in undernourished rats were of a reversible nature or not undernourished rats were nutritionally rehabilitated from birth and from early postnatal ages.

Sciatic nerves of rats rehabilitated from birth to 90 days of age showed a partial restoration of the calibre spectrum when compared with age-matched control rats. This partial restoration of the calibre spectrum of the sciatic nerve is in accordance with the ultrastructural findings of Hedley Whyte (1973) which showed that the reduced axonal circumference and number of myelin lamellae in sciatic nerves of undernourished rats were only partially recovered by subsequent rehabilitation.

Ventral roots of previously undernourished rats rehabilitated from various ages between birth and 40 days up to adult age showed when rehabilitation was initiated before the period of accelerated fibre growth (around the age of 20 days) a complete restoration of the calibre spectrum. Contrary to this, when rehabilitation was initiated after the period of accelerated fibre growth no restoration of the calibre spectrum was observed however the calibre spectrum was similar to that of age matched undernourished rats. These findings are in agreement with the hypothesis launched by Dobbing (1968) concerning the brain growth, that the vulnerable period coincides with the time of accelerated growth.

In contrast to the complete recovery of the ventral root after early rehabilitation, nutritional rehabilitation even if initiated at birth, showed only minimal effects on the calibre spectrum of the dorsal root. When rehabilitation was initiated before 20 days of age i.e. just before weaning thin fibres increased slightly in number. When rehabilitation was initiated after this age a slight increase in number of thick fibres was observed whereas thin fibres did not show any increase in number when compared with age-matched undernourished rats. The rehabilitation did not change the location of the maxima characteristic for undernourished rats and did not result in statistically significant changes in fibre distribution compared with the calibre spectrum of undernourished rats. It is thus obvious that undernutrition causes an early initiated irreversible damage to the dorsal root.

Looking at the growth velocity curves of the sciatic nerve and its roots of both normal and undernourished rats (figures 11, 18b and 19b) it can be seen that the development of nerve fibre calibres show discontinuous courses. This discontinuous increase in nerve fibre thickness may reflect changes in the synthesis of proteins essential for the growth of neurons. This hypothesis is supported by observations stating that the rate of increase of substances necessary for protein synthesis, e.g. neuronal RNA (Haltia, 1970) and those included in the free amino acid pool (Miller 1969) also show discontinuous courses during development.

Comparing the occurrence of the phase of the most accelerated growth in undernourished and control rats principal differences can be noticed between the

ventral root on one hand and the dorsal root and the sciatic nerve on the other hand. In the ventral root the phase of fastest growth in undernourished rats coincides chronologically with that in control rats. In the dorsal root and the sciatic nerve this phase was delayed as compared with that of respective nerves of control rats. In all three nerves of undernourished animals the ultimate growth achievement represented by the area enclosed between the growth velocity curve and the time axis was reduced. This is obvious when comparing the areas below the curves representing the nerves of undernourished rats with the areas below the curves representing the nerves of control rats.

According to Dobbing and Sands (1971) biosynthetic mechanisms involved in growth phenomena may be present only at the time of growth spurt. When this phase is passed achieved deficits in growth will persist. In the present study we have seen that undernutrition caused persistent deficits in the dorsal root and partially persistent deficits in the sciatic nerve whereas the calibre spectrum of the ventral root could easily be restored by early initiated rehabilitation. The irreversible effects on the dorsal root and the partially irreversible ones on the sciatic nerve could however not be prevented even if rehabilitation was initiated long before the phase of accelerated growth. The partial restitution of the nerve fibre calibres of the sciatic nerve of rehabilitated rats is presumably due to the summation of the in principle different effect of rehabilitation observed between the fibres of the ventral and the dorsal spinal roots, since they both merge into the mixed sciatic nerve.

The delay in the phase of fastest growth in the dorsal root and the sciatic nerve may be responsible for the retarded appearance of defined maxima of large fibres in undernourished rats.

The reason for the difference in occurrence of the rapid growth phase noticed between the ventral and dorsal roots of undernourished rats in relation to the occurrence of respective phases in control rats can only be a matter of speculation. It is obvious that diet provides the necessary precursor pools, like the amino acid pool, for macromolecular synthesis and that a reduction in pool size may affect the synthetic rate. This may be affected differently in anterior horn motor neurons and spinal ganglion cells, since possible disturbances in enzyme pattern, in energy metabolism or in hormonal regulation may interfere differently with amino acid incorporation and macromolecular synthesis in these cells (cf. Müller 1969) and therefore be responsible for the different effect of undernutrition upon fibre growth in ventral and dorsal roots.

V SUMMARY

The effect of experimental pre- and postnatal undernutrition on the development of nerve fibre calibres of the sciatic nerve the ventral and dorsal roots of the fifth lumbar segment of the rat was systematically investigated. The main findings were as follows.

- 1 Undernutrition initiated early in life causes deficits in the nerve fibre calibre growth in the sciatic nerve and its roots. In all nerves investigated the thick fibres were more affected in calibre growth by undernutrition than the thin fibres.
- 2 The impairment in nerve fibre calibre growth of the dorsal root proved to be irreversible after early initiated nutritional rehabilitation. In the sciatic nerve the fibre growth was partially restored and in the ventral root it was completely restored after nutritional rehabilitation.
- 3 The peaks of accelerated growth of the dorsal root and the sciatic nerve of undernourished rats were delayed as compared with those of control rats. In the ventral root of undernourished rats the peak of accelerated growth occurred at the same age as in control rats.

Thus experimental undernutrition induces reversible deficits in the calibre growth of the ventral root fibres whereas in the dorsal root the fibre calibre growth is permanently distorted by undernutrition. In the sciatic nerve the impairment of calibre growth caused by undernutrition is partially reversible.

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VII TABLES

Table Ia. Fibre distribution in sciatic nerves of normal rat 5-40 days old.

Age in days	Animal nr	0,42-0,80	0,80-1,19	1,19-1,57	1,57-1,96	1,96-2,33	2,33-2,72	2,72-3,10	3,10-3,49	3,49-3,87	3,87-4,25	4,25-4,63	4,63-5,02
5	244/1	4	48	173	644	474	2,9	110	38	8	3		
	244/2	4	42	228	561	389	190	62	17	4			
10	289/2	0	0	17	279	373	204	143	98	80	54	12	6
	289/5	0	0	19	233	416	251	236	151	86	61	22	6
15	289/7	0	0	44	485	490	284	185	96	50	7	2	
	222/3	0	2	68	428	360	287	290	262	253	182	92	34
20	222/4	0	0	18	202	274	213	280	273	269	194	130	62
	222/5	0	1	115	521	282	218	245	208	192	179	114	88
25	221/4	0	4	17	72	204	215	240	298	305	252	176	136
	221/5	0	8	40	87	211	282	287	236	329	261	264	223
30	221/6	0	1	29	140	302	402	423	433	362	253	178	114
	223/5	0	6	33	129	224	166	191	257	264	276	234	205
35	223/8	0	0	26	109	212	139	144	139	190	191	174	179
	213/5	2	6	11	77	278	264	238	221	211	229	183	185
40	213/6	1	12	31	133	225	183	149	146	168	242	201	218
	213/7	0	1	18	103	353	305	268	232	248	277	230	239
45	222/2	0	0	6	77	127	121	122	126	126	133	141	175
	227/6	0	0	21	111	99	85	86	134	120	163	168	246
50	227/7	0	0	17	91	95	98	113	164	127	120	148	129

Fibre distribution in sciatic nerves of normal rat 60-180 days old

Age in days	Animal nr	1,26-1,83	1,83-2,42	2,42-2,98	2,98-3,56	3,56-4,14	4,14-4,70	4,70-5,28	5,28-5,86	5,86-6,43	6,43-7,01	7,01-7,58	7,58-8,16
60	221/3	8	194	274	274	231	216	185	221	235	245	162	87
	221/7	76	201	241	230	217	278	793	324	263	167	99	52
90	221/8	4	169	17	249	27	250	242	250	226	216	137	74
	200/1	11	48	113	146	162	136	180	151	171	172	218	229
120	200/2	11	44	127	191	237	200	196	214	234	257	315	256
	200/3	14	42	78	162	167	154	168	139	157	214	219	221
150	139/2	9	94	121	123	139	133	115	137	122	137	147	158
	139/6	15	101	174	2	251	271	210	209	225	250	251	281
180	215/5	0	6	22	57	121	146	137	145	129	111	121	136
	243/1	4	85	115	117	174	163	164	168	148	145	186	213
210	243/2	4	115	161	142	210	175	171	179	05	179	281	297
	243/3	1	56	87	14	148	147	161	154	148	148	147	151
240	245A/2	3	25	100	115	144	134	125	135	119	154	175	190
	245A/6	3	27	92	134	135	109	115	125	116	140	153	176
270	245A/7	10	64	170	221	245	229	232	258	256	254	265	268

VII TABLES

Tabk 1a Fibre distribution in sciatic nerves of normal rats 5-40 days old

Age in days	Animal nr	0,42-0,80-	0,80-1 19	1 19-1,57	1,57-1 96	1 96-2,33	2,33-2,72	2,72-3,10	3 10-3 49	3 49-3,87	4
5	244/1	4	48	173	644	474	229	110	38	6	
	244/2	4	42	228	561	389	190	62	17	4	
10	289/2	0	0	17	279	373	204	143	98	80	
	289/5	0	0	19	233	416	251	236	151	8	
	289/7	0	0	44	485	490	284	185	96	50	
15	222/3	0	2	68	428	360	287	290	262	253	18
	222/4	0	0	18	202	274	13	280	273	269	194
	222/5	0	1	115	521	282	218	245	208	192	174
20	221/4	0	4	17	72	04	215	240	298	305	25
	221/5	0	8	40	87	211	282	287	236	329	261
	221/6	0	1	29	140	302	402	423	433	362	253
25	223/5	0	6	33	129	224	166	191	257	264	276
	223/8	0	0	26	109	212	139	144	139	190	191
30	213/5	2	6	11	77	278	264	238	221	11	229
	213/6	1	12	31	133	225	183	149	146	168	24
	213/7	0	1	18	103	353	305	268	232	248	277
40	222/2	0	0	6	77	127	121	122	126	126	133
	16	0	0	1	111	99	85	86	134	120	163
	7	0	0	17	91	95	98	113	164	127	120

Fibre distribution in sciatic nerves of normal rats 60-180 days old

Age in days	Animal nr	1 26-1 83	1 83-2 4	2 4-2 98	2 98-3 56	3 56-4 14	4 14-4 70	4 70-5 28	5 28-5 86	5 86-6 43	6 43-7 01	7 01-7 7
60	221/3	8	194	274	274	231	216	185	221	235	245	162
	221/7	6	201	241	230	217	278	293	324	263	167	99
	221/8	4	169	217	249	227	250	242	250	226	216	137
90	200/1	11	58	113	146	162	136	180	151	171	172	218
	200/2	11	44	127	191	237	200	196	14	234	257	315
	200/3	14	42	78	162	167	154	168	139	157	214	219
120	139/2	9	94	121	123	139	133	115	137	122	137	147
	139/6	15	101	174	222	251	271	210	209	225	250	251
	215/5	0	6	22	57	121	146	137	145	129	111	121
150	243/1	4	115	115	117	174	163	164	168	148	145	186
	243/2	4	115	161	142	230	175	171	179	205	179	281
	243/3	1	56	87	1 4	148	147	161	154	148	148	147
180	245A/2	3	25	100	115	144	134	125	135	119	154	175
	45A/6	3	27	92	134	135	109	115	125	116	140	153
	245A/7	10	64	170	221	245	229	232	258	256	254	265

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 407

Physiology
of
Swimming Man

BY

INGVAR HOLMÉR

STOCKHOLM 1974

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 40

From the Department of Physiology Gymnastik och idrotts högskolan, Stockholm, Sweden
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Physiology of Swimming Man

BY

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STOCKHOLM 1974

Why is it easier to swim in the sea than in rivers? Is it because the swimmer swims by continually supporting himself against the water and we can gain more support in water which contains more solid matter? For sea water contains more solid matter than river water. For it is denser and more capable of offering resistance.

Aristotle (384-322 B.C.) Problems XXXII, 13

Why do extremities become most chilled? Is it because they are so narrow? The channels in them, because they are narrow, have but little blood, so that they contain but little heat, for blood is hot.

Aristotle (384-322 B.C.) Problems VIII, 5



Fig 1 Determination of oxygen uptake during swimming in the swimming flume

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Physiology of Swimming Man

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av matematisk-naturvetenskapliga fakulteten
vid Stockholms universitet för avläggande av filosofie doktorexamen kommer att
offentligen förvaras i Wenner-Grens Instituts föreläsningssal,
fredagen den 19 april 1974 kl. 10.00

AV

INGVAR HOLMÉR

Fil. kand.

STOCKHOLM 1974

The present publication is based on studies reported in the following paper

- I Holmér I Oxygen uptake during swimming in man J Appl Physiol 33(4) 502-509 1972
- II Holmér I and P-O Åstrand Swimming training and maximal oxygen uptake J Appl Physiol 33(4) 510-513 1972
- III Holmér I, A Lundin and E O Eriksson Maximum oxygen uptake during swimming and running by elite swimmers J Appl Physiol 1974 (in press)
- IV Holmér I, E M Stein, B Saltin, B Ekblom and P-O Åstrand Hemodynamic and respiratory responses compared in swimming and running J Appl Physiol 1974 (in press)
- V Döbeln W von and I Holmér Body composition, sinking force and oxygen uptake of man during water treading J Appl Physiol 1974 (in press)
- VI Holmér I Mechanical efficiency of breaststroke and freestyle swimming (for publication 1974)
- VII Holmér I Aerobic energy output and efficiency of different competitive swimming strokes (for publication in 1974)
- VIII Nadel E R, I Holmér, U Bergh, P-O Åstrand and J A J Stolwijk Energy exchange of swimming man J Appl Physiol 1974 (in press)
- IX Holmér I and U Bergh Metabolic and thermal responses to swimming in water at varying temperatures (for publication in 1974)

In the text these papers will be referred to by Roman figures I-IX

INTRODUCTION

A number of fundamental criteria must be considered in order to obtain reliable measures of the body's reaction to a given rate of work. (i) Measurements must be made during work. (ii) Measurements shall be made where applicable in steady-state conditions, i.e. when various physiological functions have adapted to the load. (iii) The work procedure during measurements must coincide with the natural exercise to the greatest possible extent. (iv) It shall be possible to make an accurate determination and reproduction of the rate of work.

Different types of ergometers have been designed for different types of work in order to satisfy these criteria.

Up to 1970, most of the studies made on swimming, the emphasis has been on a determination of oxygen uptake, heart rate, and respiratory function. Measurements in water and a swimmer's movements and propulsion entail complications which have prevented complicated studies.

Swimming in a pool or a lake creates difficulties in reproducing swimming speed, and the turns in a pool introduce another disturbing factor. Tethered swimming results in hydromechanical conditions which differ from those in free swimming, clearly reducing the applicability of obtained data to free swimming.

The first scientific thesis on swimming was written by DuBois Raymond (1905) who calculated energy metabolism during swimming on the basis of measurements from other work forms and a number of mechanical considerations. However, the data were criticised by Liljestrand & Stenstrom (1919) who measured oxygen uptake during swimming in a lake. Since that time a number of studies have been made using different experimental designs and methods. The duration of work in some of the latter studies was too brief, i.e. 1-2 min, to permit any measurement of energy requirements in steady state conditions (Karpovich & Millman 1944; Goodwin & Cumming 1966; Adrian et al. 1966). In other studies breath-holding during swimming in combination with the determination of oxygen uptake during the recovery phase following submaximal (Karpovich & Millman 1944) and maximal work (Karpovich & Millman 1944; Adrian et al. 1966; Kili-souris 1968) was used in order to estimate energy requirements. This technique has been strongly questioned (Christensen et al. 1960). Oxygen debt is probably used to pay off more than the energy debt incurred by anaerobic processes (cf. Åstrand & Rodahl 1970). Furthermore the energy expenditure required to eliminate lactate formed during exercise is eventually twice the energy yielded from the breakdown of glycogen into lactate (Krebs 1964).

A number of workers have also measured oxygen uptake in steady state condition during swimming in the sea (Pugh & Edholm 1955) and in a pool (Fredrik-

sen 1945 Hemmingsen 1957 Andersen 1960 Åstrand & Saltin 1961 b Åstrand et al 1963 Klissouras 1968 McArdle et al 1971)

None of these swimming studies were able to satisfy the four criteria specified at the beginning of this paper. Therefore the results obtained in these studies have limited validity (also see the comments in I IX). Despite more or less serious shortcomings in the methods used the cited studies have still managed to obtain some information which has proved to possess general validity. (i) Trained swimmers are distinguished by a lower oxygen uptake at a given submaximal speed than untrained swimmers. (ii) The freestyle (front crawl) and backstroke are more economical swimming style than the breaststroke and butterfly at any given speed. (iii) Leg kicks are less efficient in the freestyle than arm strokes alone. (iv) The highest values for pulmonary ventilation and heart rate during maximal swimming are lower than in maximal running, cycling and walking.

The development of a swimming flume (Åstrand & Englesson 1972) made it possible to reproduce swimming speeds and eliminated a number of technical problems associated with measurement. The four specified criteria could be satisfied by the present investigation and a relatively detailed analysis of the physiological response to swimming was performed.

The purpose of the present study was thus to
devise and investigate methods for the determination of different variables during swimming in a swimming flume;
study circulatory and respiratory functions during swimming
compare physiological response during swimming with the corresponding reactions during running and cycling
examine the manner in which the body's heat balance and oxygen uptake are affected by work in water at varying temperatures
determine oxygen uptake during swimming at different speed and with different swimming styles used by swimmers of varying capability and in varying states of physical condition;
analyse mechanical work during swimming and calculate mechanical efficiency.
It should be emphasized that the special problems related to underwater swimming were not considered.

METHODOLOGICAL CONSIDERATIONS

Abbreviations and symbols

The abbreviations and symbols in this work with a few minor exceptions are applied according to the guidelines devised by the Glossary Committee of the International Union of Physiological Sciences (See Glossary Committee of the IUPS 1973 a, b)

Abbreviation

or Symbol

Term (unit)

a-v difference	arterio-venous difference
C	convective heat transfer ($W m^{-2}$)
$^{\circ}C$	degree Celsius
ERV	expiratory reserve volume (l)
h_c	Convective heat transfer coefficient ($W m^{-2} ^{\circ}C^{-1}$)
IRV	inspiratory reserve volume (l)
J	Joule
kp	kilopond (=9.81 N)
$kpm min^{-1}$	kilopondmeter per minute (=0.163 W)
$l min^{-1}$	liter per minute
M	metabolic free energy production (W)
$m s^{-1}$	meter per second
mm Hg	millimeter of mercury column (=1 Torr)
N	Newton
n	number of subjects determinations at work efficiency (%)
P	pressure (mm Hg)
P_{aO_2}	arterial oxygen pressure
\dot{Q}	cardiac output ($l min^{-1}$)
R	respiratory quotient (also RQ)
	correlation coefficient
RML	mean respiratory level (l)
RV	residual volume (l)
S_{aO_2}	arterial oxygen saturation (%)
SD	standard deviation
SE	standard error of the mean
SMR	standard metabolic rate (W)
T	esophageal temperature ($^{\circ}C$)
T_{ms}	muscle temperature ($^{\circ}C$)
T_{sk}	mean skin temperature ($^{\circ}C$)
T_w	water temperature ($^{\circ}C$)

TLC	total lung capacity (l)
V_E	pulmonary ventilation (l min ⁻¹)
$V_E V_{O_2}$	ventilatory equivalent
$\dot{V}O_2$	oxygen uptake (l min ⁻¹)
V_T	tidal volume (l)
\dot{V}	Watt (l J s ⁻¹)
W	rate of work (W)
W_F	sinking force of an immersed body (kp)
W_W	weight in water (kg)
<	less than

Subjects

Women and men of varying proficiency and varying degrees of swim training were used as subjects. Table I provides a brief description of the groups of subjects in the different studies. (See each respective study for a more detailed description.) As the Table shows, their maximal oxygen uptake was usually high in relation to the general population (Åstrand 1952, Åstrand 1960) and most of the subjects were well trained. This made it possible to perform a number of trials at different speeds at each session, including two maximal efforts in one day in some cases, without any significant change in measured values as compared to trials on different days.

All subjects were volunteers and trained prior to the actual measurements so that they were familiar with the equipment and with the experimental designs.

Statistics

Conventional methods were used for the statistical processing in order to determine the mean difference and the t test (Armitage 1970). Calculation of multiple regression were performed using the least squares method. Computer program BMDO2R (Health and Science Computer Facilities UCLA) was used as primary multiple regressor. The error of the method has been specified in the standard deviation for a single experiment calculated from double determinations according to the following equation:

$$\text{Error of method} = \sqrt{\frac{\sum (d - \bar{d})^2}{n - 1}}$$

in which d is the difference between double values, \bar{d} = the mean difference and n = the number of determinations.

Table 1 Characteristic of subject groups in the various studies. Mean values and ranges are given. Mixed group refers to subjects of varying skill and training status in swimming

Study	Age yrs	Height cm	Weight kg	Maximal oxygen intake l min ⁻¹		Comments
I	3	174	66	2.89	3.24	Mixed group
		162-181	57-73	2.28-3.63	2.51-3.61	
	8	180	74	4.14	4.67	Mixed group
		179-183	71-74	3.32-5.08	4.03-5.59	
II	12	165	54	2.96	3.17	Trained swimmers
		145-174	33-64	1.59-3.76	1.81-3.76	
	1 (11)	180	73	3.63	3.61	Identical twins; L = elite swimmer
		181	89	2.82	3.56	
III	11	170	66	3.42	3.64	Elite swimmers
		167-175	57-71	2.94-3.76	3.25-4.04	
	12	184	78	5.05	5.38	Elite swimmers
		175-192	69-85	4.04-5.93	4.73-6.42	
IV	5	181	73	3.79	4.54	Mixed group; all trained
		176-185	62-89	3.08-4.50	3.69-5.57	
	11	169	62	6.2	6.2	Trained swimmers
		161-179	54-81	5.4	5.4	
V	4	179	75	7.5	7.5	Trained swimmers
		174-182	66-87	6.6	6.6	
	3	181	76	4.74	4.74	Elite swimmers
		175-188	63-84	4.05-5.19	4.05-5.19	
VII	19	180	74	4.49	4.49	Elite swimmers
		170-189	56-89	3.38-5.74	3.38-5.74	
	3	179	76	4.13	4.13	Trained swimmers
		174-182	66-87	3.89-4.55	3.89-4.55	
IX	5	178	69	3.72	4.27	Mixed group; all trained
		165-184	52-78	3.09-4.19	3.24-4.73	

Methods

In this section a summary is provided of the experimental methods used. A more detailed description will be found in the separate reports.

Swimming was performed in a swimming flume (Stenberg Flygt AB, Solna, Sweden) in which water could be made to circulate at speeds from 0.02 to 2.0 m s^{-1} (Åstrand & Englesson 1972). Reproducibility for a given water velocity amounted to 1.4% the variation in speed for the water which a swimmer normally came in contact with was less than 0.02 m s^{-1} . The task of the subject was to remain on the spot by swimming (Fig. 1.2).

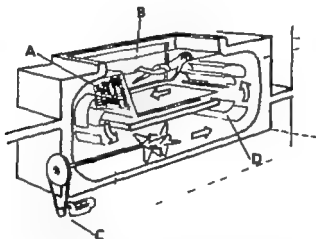


Fig. 2. Drawing of the swimming flume. A = safety net. B = $2.5 \times 1.5 \text{ m}$ window. C = electric motor. D = vane guided bends (from Åstrand & Englesson 1972).

Running took place on a motor driven treadmill whose speed and inclination could be adjusted. Cycling was performed on an electrically or mechanically braked bicycle ergometer.

Oxygen uptake was calculated from expired air samples collected in Douglas bags and analyzed for O_2 and CO_2 by the Haldane or the Scholander technique after volume determination by balanced spirometer. Blood lactate levels were determined with the Barker-Sommerson method as modified by Ström (1949). Cardiac output was established using the dye-dilution method with indocyanine green (Cardiogreen®) as the indicator (IV). Heart rate was calculated on the basis of ECG records. Intra-arterial blood pressure was measured with an Elema-Schönander pressure transducer. O_2 content, O_2 saturation and CO_2 content of the blood

were determined using a van Slyke device while O_2 and CO_2 pressures and pH were established according to Astrup's technique (IV). Esophageal temperature, muscle temperature and skin temperature were determined with thermocouples and heat flow was measured with Hatfield-Turner heat flow discs (VIII-IX). Mean skin temperature and the average heat flow from the body were determined as a weighted mean value of four and five measurement points respectively on the body (VIII).

The body's sinking force and water drag were measured with a wire strain gauge and recorded using a Honeywell recorder (V). A method devised by di Prampero et al. (1974) was applied in the determination of the body's drag during swimming (VI-VIII). Mechanical efficiency was calculated as the ratio between mechanical work and the energy metabolism during work minus standard metabolic rate (VI):

$$\eta = \frac{W}{M \cdot BMR} \times 100 (\%)$$

The weight of each subject in water with the body completely submerged, the residual volume of the lungs and the body's density were determined according to a method described by von Döbeln (1956). (For details on these and following anthropometric measurements see I and V). The weight of adipose tissue and the average thickness of subcutaneous fat were calculated on the basis of skinfold measurements from the abdomen and thigh (Hermansen & von Döbeln 1971). Skeletal weight was calculated from measurements of the width of the right and left femoral condyle, the right and left radioulnar width and body height (von Döbeln 1959). Total potassium was determined by means of whole body counting employing gamma spectrometry in a lead chamber (von Döbeln & Lindell 1964). The weight of fat-free soft tissue was calculated from the amount of potassium.

Determination of pulmonary volumes and certain pulmonary functions were made with the aid of a Bernal spirometer (V).

Comments

In every case each sensor and the recording equipment were calibrated in conjunction with each individual trial session. The error of the method for a few measured variables is shown in Table 2. This includes both error of measurement and biological variations and was calculated from two determinations made on different test occasions or on the same (cardiac output). The error of method for determination of oxygen intake and cardiac output were in close agreement with values reported previously (Ekblom 1969; Kilbom 1971; Eriksson 1971).

Oxygen intake values stabilised with and without arterial and venous catheters during maximal swimming and running did not differ significantly (IV). Swimming in the swimming flume entailed prerequisites for attaining a maximum oxygen uptake as high as in swimming in a pool (Table 2, III).

Table 2 Error of the method in determination of oxygen uptake, cardiac output and correlation coefficient and difference between means for some physiological variables measured under different conditions

	n	Mean value	Error of method	Difference between means	Correlation coefficient
<u>Oxygen uptake ($l \cdot min^{-1}$)</u>					
Submaximal swimming	59	2.76	2.9		
Maximal swimming	47	3.78	2.1		
<u>Cardiac output ($l \cdot min^{-1}$)</u>					
Submaximal swimming	11	17.3	5.4		
Maximal swimming	5	26.0	3.6		
Submaximal running	18	18.6	5.2		
Maximal running	7	28.4	4.2		
<u>Drag (kp)</u>					
Passive towing	61	3.51	3.9		
During swimming	6	2.92	5.7		
<u>Oxygen uptake ($l \cdot min^{-1}$) during maximal swimming in flume vs pool</u>					
	7			0.02	0.99
<u>Oxygen uptake ($l \cdot min^{-1}$) during swimming and running with and without catheters</u>					
	10			0.01	0.99
<u>Arterial O_2 saturation (%) with and without valve</u>					
	12			1.6	0.89
<u>Arterial O_2 pressure (mm Hg) with and without valve</u>					
	17			-0.2	0.91

Procedure

Details of the experimental design in the different studies are reported in each respective article. Only some general principles will be discussed in this section.

At each test session, work was either performed on a treadmill, bicycle ergometer or in a swimming pool. As a rule, a series of determinations were made at light, moderate, heavy and maximal intensities.

Light/moderate/heavy work intensities: The total duration of exercise at each submaximal intensity usually amounted to 5-7 min. Heart rate was recorded each minute, oxygen uptake during the final 1-2 min and blood samples for lactate assay

were taken 1-2 min after the conclusion of exercise and occasionally 5-10 min after exercise in order to guarantee attainment of peak values.

Maximal work intensities. The maximal oxygen uptake specified for a subject generally consisted of a mean value for determinations made at a few days interval. In the determination of cardiac output and in conjunction with work in water at different temperatures, the subjects' maximal oxygen uptake was determined in trials prior to the start of the actual study. In some exceptional cases there was no opportunity of obtaining two independent determinations of a subject's maximal oxygen uptake and the following procedure was then applied: Maximal running at a constant speed was used and the inclination at the start was 3° (5-25%) being increased by 1.5° (2-60%) every third minute. Maximal swimming water speed was increased by 0.1 m s⁻¹ every other minute, starting with a speed which was 10-20% lower than the maximal speed calculated for each subject (III). In both modes of exercise work was performed until complete exhaustion.

Comments

The average difference in oxygen uptake (0.02 l min⁻¹) between two determinations made with 19 subjects who performed maximal swimming at an interval of a few days was not significant. The difference in swimming speed amounted to 0.08 m s⁻¹ (7.7%; range 4.4-20.0%). The duration of exercise for the test at a somewhat lower speed was 5:27 min (range 3:35-7:00) and for the test at a somewhat higher speed 3:23 min (range 2:20-4:40). Thus maximal oxygen uptake could be achieved with a work duration of 3-4 min or 5-7 min during swimming (cf. Åstrand & Saltin 1961a). The levelling-off criterion for the determination of maximal oxygen uptake was used as described by Ekblom (1969). In this context the maximal swimming speed was stated as the lowest one at which the swimmer achieved maximal oxygen uptake.

RESULTS AND DISCUSSION

This section is divided into four parts: the first of which provides a brief description of the mechanical load factors in swimming. The second part deals mainly with the metabolic and cardio-respiratory responses to swimming in general. The third part elucidates the heat exchange and the metabolic response to swimming in water at different temperatures. The section is completed with a discussion of the various swimming styles from point of view of efficiency and maximal energy output and with concluding remarks.

Mechanical load factors

A person with some part of his body in the water and with unimpeded airways is affected by a sinking force whose magnitude depends on a number of anthropometric variables (such as the weight of muscle and adipose tissue) (Fig. 3).

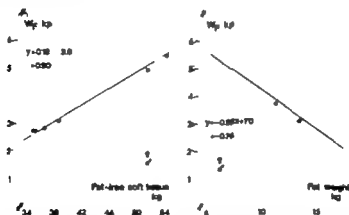


Fig. 3 The body's sinking force in relation to fat-free soft tissue (left) and fat weight (right). Regression line equation and correlation coefficient stated. (From V)

the weight of body parts above the surface of the water and the volume of air in the lungs (Fig. 4). If the lungs are filled with air to a maximum and the body is submerged so that only a small part is out of the water, most people should be able to maintain a state of equilibrium in the water without the use of active muscular work. During swimming and water treading, however, a somewhat larger part of the body is normally above the water and the lungs are only partly filled with air. Therefore the body is acted on by a sinking force which must be counteracted with muscular force. If the exercise is not very vigorous (i.e. partly anaerobic) oxygen

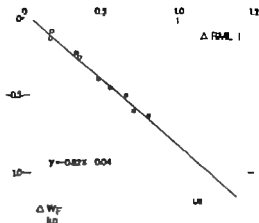


Fig 4 Change in sinking force in relation to change in respiratory mean level. Regression line and equation entered. (From V)

ptake is the best measure of the extra energy yielded by the muscles.

Oxygen uptake during water-treading proved to be directly related to the body's sinking force (Fig 5) (a relationship which was also described by Hemmingsen 1957). Individuals with a large amount of body fat, relatively speaking, were acted on by a smaller sinking force than might be anticipated from body weight (Fig 3). This circumstance generally led to a somewhat higher body position in water for girls than for boys.

An additional force component arises during swimming, since when a body moves through a fluid a force acts backwards on it, resisting its motion. This force is known as drag and its amount depends on the fluid and on the size, shape,

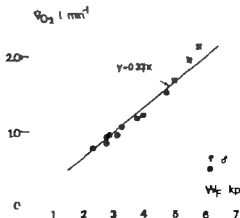


Fig 5 Oxygen uptake during water-treading in relation to sinking force. Regression line and equation entered. The correlation coefficient was 0.96. When W_F was increased by loading subjects with known weight, the increase in $\dot{V}O_2$ followed the regression line. (From V)

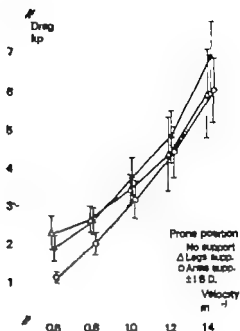


Fig 6 Drag during passive towing of the body in three positions in relation to water speed. Mean value ± 1 SD for seven male subjects (From VIII)

and speed of the body. Individuals with a large body surface are exposed to greater water drag (Karpovich 1933, Alley 1952, Hansen 1955, Counsilman 1968). The body's position in water and during the different phases of the swimming stroke affects the amount of water drag (Counsilman 1955, Kent & Atha 1971).

Drag during passive towing of the body through flowing water increased exponentially as the water speed increased (Fig 6). At higher speeds, the water exerted a lift on the lower parts of the body (hydrodynamic lift) and therefore the body assumed a more horizontal position at high speeds than at low speeds. If the legs were lifted into a horizontal position using a cork plate at high speed, drag was further reduced (Fig 6). When the subject was swimming, drag in breaststroke and freestyle was calculated to be approximately 2 and 1 1/2 times greater respectively than in passive towing (Fig 7).

Thus total drag during swimming was greater than in passive towing. Corresponding results have been reported by other authors (di Prampero et al 1974).

In this context stroke is defined as a complete work cycle of arms or legs or both. Style refers to the type of swimming e.g. freestyle (front crawl), backstroke, breaststroke or butterfly (dolphin stroke).

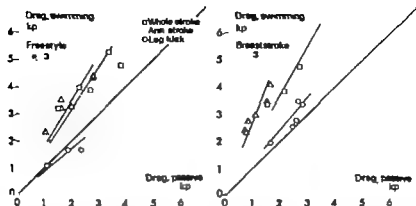


Fig 7 Drag during swimming with the whole stroke and with arm or legs alone in the freestyle (left) and breaststroke (right) compared to passive drag at corresponding velocities (From VL)

Since the lower part of the body is denser than the upper part there is a more pronounced tendency for the legs to be drawn down when the body assumes a horizontal position in water. In order to attain the most favourable position from the point of view of drag a certain amount of work must be performed with the legs to counteract this downward pull on the legs. The significance of this force on the legs was not examined in detail in this study and therefore not considered when calculating the mechanical work.

Therefore mechanical work during swimming has been expressed as water drag multiplied by the distance swum (VL). Mechanical efficiency can be calculated by measuring energy metabolism during swimming and at rest.

Metabolic and cardio-respiratory responses to swimming

Energy metabolism

During swimming at low speed the measured oxygen uptake was of the same magnitude as during water-treading (Fig 8). Interindividual differences in the body's sinking force were reasons for the noticed variations in energy expenditure at low speeds. Thus a better measure of the efficiency of swimming work was obtained when oxygen uptake was corrected for body weight in water (Fig 8). A similar conclusion was presented by Hemmingsen (1957). Water drag increases by about the square of the speed (V^2) and as mentioned a more horizontal body position is assumed at higher speed. The result is that drag at high speeds constitute the main loading factor.

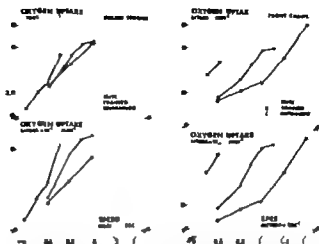


Fig 8 Oxygen uptake in relation to speed during breaststroke (left) and freestyle (front crawl) (right) swimming by three subjects selected so as to represent three training categories. In the lower half of each graph oxygen uptake is corrected for subject weight in water (From I)

Oxygen uptake displayed a linear increase or a slightly exponential increase as swimming speed increased (Fig 9). At the highest speeds oxygen uptake levelled off. This means that a maximal oxygen uptake was achieved by each subject at a given speed and was not exceeded even if the swimming speed was increased somewhat (Fig 9).

The oxygen uptake at a given submaximal speed varied among subjects. Some of these interindividual variations could be explained by differences in water drag (Fig 10).

The increase in oxygen uptake as submaximal speed increased was governed both by the increased drag as well as the swimming style and swimming technique depending partly on the degree of training. A more detailed discussion of the significance of these factors will follow.

At a given submaximal oxygen uptake blood lactate tended to be lower in trained than in untrained swimmers. Trained swimmers were able to work at a relatively high oxygen uptake (60-70%) in relation to their maximum without any elevation in blood lactate (I, VII).

Swimming Versus running

An examination of the physiological response at a given oxygen uptake during swimming is of considerable interest as the exercise medium and the position of the

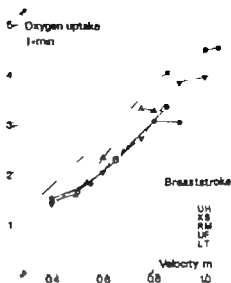


Fig 9 Oxygen uptake displayed linear or exponential increase as swimming speed increased and levelled off at higher speeds. This levelling off was used with a few exceptions to designate maximal oxygen uptake but was not generally included in the graphs. (From IV.)

body is so different in this form of exercise than in e.g. running and cycling. The present study made a detailed analysis of the circulatory and respiratory response during the breaststroke. The following results were considered in selecting this swimming style:

1. The oxygen uptake attained in maximal breaststroke swimming was at least as great as in the other swimming styles (I, III, VII).
2. Maximum load on the swimmer is achieved at a lower swimming speed than in other swimming styles (VII).

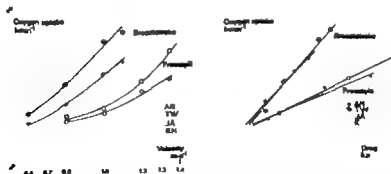


Fig 10 Oxygen uptake for two equally successful breaststroke and freestyle swimmers respectively in relation to swimming speed (left) and power drag at corresponding paces (right). Note the individual differences when oxygen uptake is related to velocity and the almost congruent relationship obtained when related to body drag. (From VII.)

3 The swimmer is relatively easy to communicate and the style presents few problems regarding interference with the probes

Running and cycling were chosen for purposes of comparison

In previous studies maximal oxygen uptake in swimming in a pool was reported lower (Åstrand & Saltin 1961 b Åstrand et al 1963 McArdle et al 1971) but in one study higher (Magel & Faulkner 1967) than in other types of exercise. The latter finding however is of limited value since swimming tests were administered later in the training season than running tests. Elite swimmers attained in swimming a maximal oxygen uptake which was significantly lower (6-7%) than in running (Fig. 11 I-III) but of about the same magnitude as in cycling (I). For untrained swimmers maximal oxygen uptake in swimming was reduced even more i.e. by about 20% compared with running and by about 10% compared with cycling (I). The explanation for the reduced maximal oxygen uptake in swimming must lie in limitations in the oxygen transport chain.

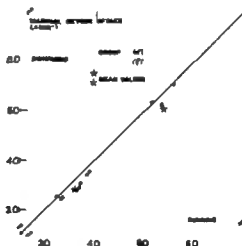


Fig. 11 Comparison of maximal oxygen uptake during swimming and running by eleven female and twelve male elite swimmers. (From III)

Respiration

For a sitting subject the vital capacity is reduced by about 10% when the body except for the head is submerged in water (Agostini et al 1966 Hong et al 1969). Most of this reduction should be ascribable to an increased blood volume in the thorax (Hong et al 1969). The increased airway resistance during submersion at rest (Agostini et al 1966) and during swimming (Deroanne et al 1971) must entail increased respiratory effort (Hong et al 1969).

Respiration in swimming is synchronized with swimming strokes and the duration of the inspiratory phase is reduced (except in the backstroke). Expiration usually takes place under the water surface (except in the backstroke) and accord

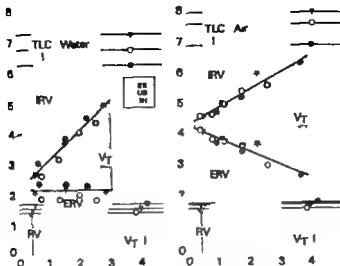


Fig 12 Total lung capacity in piratory reserve vol me residual vol me and tidal volume for three subjects in air and in water. Note that the mean respiratory level shifts towards the ERV in water and that the increase in V_T takes place exclusively by utilizing IRV (From V)

ingly against greater resistance than in air. However, in the present studies this was compensated by the use of a respiratory valve. Impaired ventilation may interfere with the transport of oxygen from the atmosphere to the pulmonary capillaries.

In water the increased tidal volume was attained exclusively through the use of the inspiratory reserve volume, in contrast to circumstances in air (Fig 12 V).

During submaximal swimming V_E , \dot{V}_E , \dot{V}_{O_2} and R were related to \dot{V}_{O_2} in the same manner as in running and cycling (Fig 13 I IV).

During maximal swimming V_E and \dot{V}_E , \dot{V}_{O_2} displayed mean values which were significantly lower than in maximal running and cycling (Figs 13 14 I III IV). Tidal volume during maximal swimming was of the same magnitude as in maximal running, while respiratory rate, apparently governed by the swimming stroke rate, was lower during maximal swimming.

Values for arterial O_2 saturation, O_2 and CO_2 contents, and O_2 and CO_2 pressure during both submaximal and maximal work were the same in swimming and running (Fig 13 IV). Blood gas values taken with and without the respiratory valve during maximal swimming were almost identical (Fig 15). Alveolar ventilation per breath

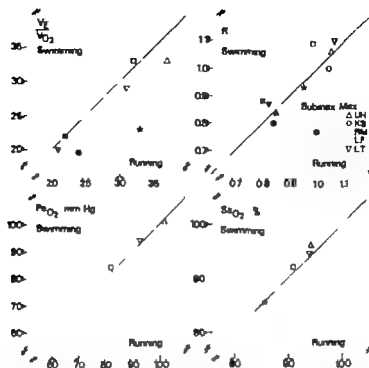


Fig 13 Comparisons of ventilatory equivalent at respiratory quotient at arterial O_2 pressure and arterial O_2 saturation in swimming and running. Submaximal swimming values interpolated. (From IV)

calculated using a modified Bohr's formula was greater during both submaximal and maximal work in swimming than in running (IV).

It was obvious that the pronounced hyperventilation usually associated with maximal exercise on land did not develop in swimming. Blood gas data however provided no indication of hypoventilation. Thus the pulmonary ventilation during maximal swimming was sufficient to saturate the blood with oxygen to the same degree as during running. Thus respiration does not appear to be restrictive to the maximal oxygen intake which can be achieved in swimming, and is no explanation of the lower maximal oxygen uptake noticed in swimming.

It should be emphasized that a number of scientists have shown that oxygen uptake during submaximal and maximal cycle work is only influenced insignificantly even when respiratory resistance is doubled (Demedts & Anthonisen 1973, Flook & Keelman 1973). Pulmonary ventilation however during maximal exercise was about 10% reduced (Demedts & Anthonisen 1973). Hence the ventilatory equivalent was lower during maximal cycling with increased external airway resistance. This is also in agreement with the respiratory response to maximal swimming.

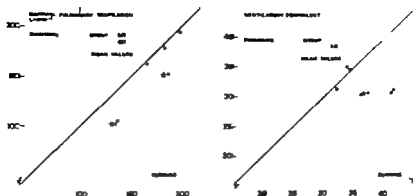


Fig 14 Comparison of maximal values for pulmonary ventilation and ventilatory equivalent in swimming and running by elite swimmers (From III)

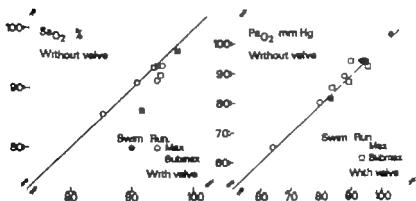


Fig 15 Arterial O_2 saturation and arterial O_2 pressure compared during swimming with and without respiratory valve (From IV)

Circulation

Circulation the next link in the oxygen transport chain has been the subject of very few detailed studies during swimming. Liljstrand & Lindhard (1919) and Dill & Faulkner (1971) measured the cardiac output during tethered swimming using the gas rebreathing technique. Saltin (1973) has reported data on cardiac output during swimming but only for one subject.

Cardiac output during swimming displayed an almost linear increase as $\dot{V}O_2$ increased and at a given submaximal $\dot{V}O_2$ it was about the same as in running (Fig 16)

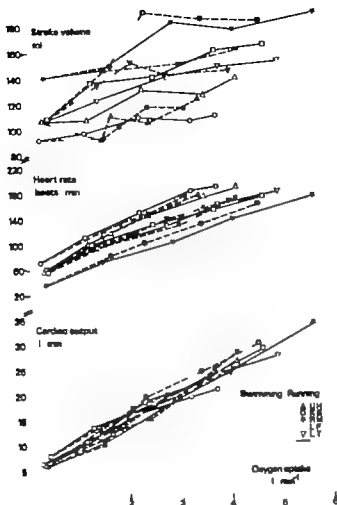


Fig 16 Stroke volume heart rate and cardiac output during swimming and running in relation to oxygen uptake (From IV)

Also heart rate during swimming displayed a linear increase related to $\dot{V}O_2$ and was of about the same magnitude at a given submaximal $\dot{V}O_2$ as in running (Fig 16)

Cardiac output and heart rate during maximal swimming were less than in maximal running (Figs 17 18). The calculated stroke volume during submaximal and maximal swimming was of about the same magnitude as in running. Stroke volume increased in both exercise forms in the transition from supine rest to light submaximal work thereafter remaining relatively constant as the rate of work increased (Fig 16)

The systemic $a-v$ O_2 difference at a given submaximal $\dot{V}O_2$ was about the same

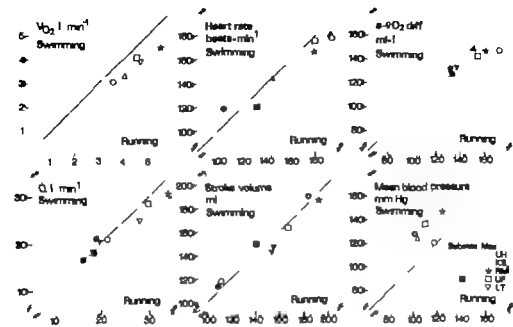


Fig 17 Val = for oxygen uptake heart rate cardiac output stroke volume arterio-venous O₂ difference and mean blood pressure compared during swimming and running Submaximal values compared at corresponding oxygen uptake (From IV)

in running and swimming but slightly lower in maximal swimming than in running (Fig 17). However, oxygen utilization in the working legs appears to have been at least as great in swimming as in running as the a-v O₂ difference in working legs was similar (IV). This discrepancy in oxygen extraction between swimming and running must reflect differences in distribution of blood flow implicating a relatively large blood flow to less oxygen consuming tissue of intestinal organs and skin in swimming. In short term maximal exercise on land priority is given to muscle blood flow which the running data in this study confirm. Data are needed on regional blood flow in swimming to explain accurately the observed differences between systemic and leg a-v O₂ difference.

The cardiac output can be limited by both central factors such as heart rate and stroke volume and by peripheral factors such as the size of the vascular bed influencing the peripheral resistance and the perfusion pressure. As mentioned in the introduction a heart rate significantly lower in maximal swimming than in maximal running cycling and walking is a well-documented phenomenon (of McArdle et al 1971) (Fig 18). It is also a well known fact that face immersion in combination with breath holding will lead to a depressed heart rate (diving bradycardia).

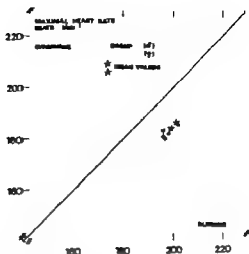


Fig 18 Maximal heart rate compared during swimming and running by elite swimmers (From III)

and this reduction is more pronounced the colder the water (Andersen 1966 Strauss 1970 Moore et al 1971) Diving bradycardia has also been demonstrated during exercise (Scholander et al 1962 Asmussen & Kristiansson 1968 Strömme et al 1970 Bergman et al 1972) However breath holding appears to be the most important cause (Brick 1966 Asmussen & Kristiansson 1968 Pavlov 1968 Strömme et al 1970) This is why criteria during swimming fail to fully satisfy the criteria associated with diving bradycardia But the effect of a diving reflex may persist This hypothesis is supported as the heart rate is somewhat lower during swimming with face immersion than in swimming with the head out of the water at a corresponding oxygen intake (Asmussen & Kristiansson 1968)

Different body positions during work i.e. whether the work is performed in an upright or recumbent position has also been shown to influence circulation The improved conditions for heart filling during diastole in a recumbent position may explain the somewhat higher stroke volume at rest and at a lighter submaximal oxygen intake compared with sitting rest and work respectively (Bevegard et al 1963 Lüscher et al 1967) However stroke volume during maximal work is the same during leg cycling and combined arm and leg cycling irrespective of body position (Stenbäck et al 1967) This is a finding in agreement with the present results: maximal swimming and running However recumbent cycling is in a way an unnatural form of exercise Swimming on the other hand normally takes place with the body in a horizontal position

During submaximal work the hydrostatic pressure counteracts the inspiratory work and in swimming mentioned expiration normally takes place in the water It is shown that postural passive breathing: man at supine rest in air entails a reduced cardiac output and that this reduction indicates an impaired venous return

which follows the increased intrathoracic pressure (Werkö 1947). However, Arbo-relli et al. (1972) demonstrated a 30% increase in cardiac output in man sub-merged in water (35°C) compared with resting conditions in air. Reamie et al. (1971) have shown that in water below 34°C cardiac output at rest and during exercise is lower than at corresponding oxygen uptake in air, probably due to cold stress. Hence, in this respect the effects of hydrostatic pressure and cold water respectively are similar.

The large heat transfer coefficient between the skin and water (see the following section) facilitates the heat transfer to the water, thereby reducing the need for skin circulation. Various demands on skin circulation due to changes in skin temperature modify the circulatory response to a given submaximal work (Rowell et al. 1969). A more detailed discussion on these factors will follow.

It is likely that the muscle mass activated in swimming, although with both arms and legs, is smaller than in running. The need for stabilization of the body in water is slight so that work for this purpose is largely eliminated. A smaller muscle mass involved in work means that a smaller quantity of oxygen can be consumed and therefore transported by the blood. Thus the smaller cardiac output and lower heart rate in maximal swimming compared with maximal running can be explained to some extent by a smaller oxygen uptake. This should be the main reason why particularly untrained swimmers had a lower maximal oxygen uptake in swimming than in running. (1)

The mean arterial blood pressure at submaximal and maximal rates of work was higher in swimming than in running (Fig. 15). The higher mean arterial blood pressure in swimming can be explained by a higher elevated external pressure and increased peripheral resistance caused by the lower temperature (26-28°C) of the skin. The difference in blood pressure may also be a result of a varying size of the activated muscle mass. Stenberg et al. (1967) showed that work at a given submaximal oxygen uptake with small muscle groups produced higher blood pressure than work with large muscle groups.

The difference between an upright and a recumbent work position also comprises a difference in perfusion pressure in working leg, depending on the effect of hydrostatic pressure (Folkow et al. 1971). Blood perfusion is therefore facilitated in running as compared with swimming. However, the elevated arterial blood pressure during swimming may result in a somewhat increased perfusion. (1V) But the importance of a raised perfusion pressure also depends on the venous pressure. At present there are no data available elucidating the difference between work forms in this respect. This circumstance plus differences in body position and exercise media make it difficult to interpret land exercise data in terms of swimming.

Effects of water at different temperatures

The circulatory response to exercise in cold water may reflect a conflict between metabolic and heat regulatory requirements which have to be satisfied simultaneously by the cardiac output. The result may be that neither of these requirements are adequately satisfied thereby impairing optimum function.

Severe cold loading leads to a reduced blood flow in peripheral tissues (cf Keatinge 1969) which could jeopardize muscle blood flow. The reduced maximal oxygen uptake observed when body temperature was lowered ($T_{re} < 37^{\circ}\text{C}$) (VII DQ) may be one effect of such a mechanism.

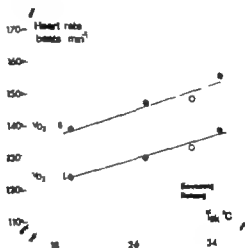


Fig. 19 Heart rate in relation to mean skin temperature at two metabolic rates. Heart rate values interpolated (from IX) and T_{sk} calculated from VIII (swimming) and from Saltin et al. (1972) (running).

The lower heart rate during submaximal and maximal swimming in the case of low central temperatures indicates a smaller cardiac output assuming stroke volume is unchanged. Rowell et al. (1969) showed that cardiac output and arterial pressure at given submaximal oxygen uptake varied in a similar manner when skin temperature changed. Nielsen (1969) calculated skin blood flow at approximately 1 l min^{-1} when $\dot{V}_{O_2} = 2 \text{ l min}^{-1}$ and $T_{sk} = 33^{\circ}\text{C}$. An increase in cardiac output from 15 l min^{-1} to 16 l min^{-1} with a constant stroke volume (140 ml) would then correspond to a 7 beats min^{-1} change in heart rate, a value of the same magnitude as the difference in swimming at 18°C and 26°C and 26°C and 34°C respectively (Fig. 19). The difference in peripheral circulation depending on various heat balance conditions can explain some of the variation in heart rate during exercise in water at varying temperatures and the difference in heart rate between maximal swimming and maximal running (Fig. 20 DQ).

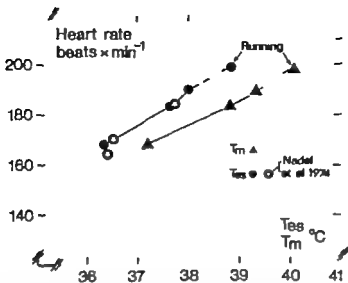


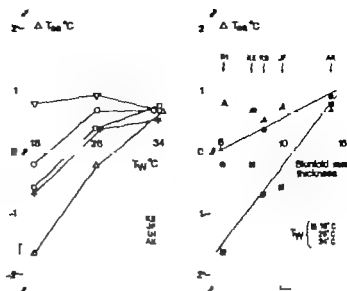
Fig 20 Heart rate during maximal swimming and maximal running in relation to oesophageal temperature and muscle temperature. Mean values for five male subjects. Data from VIII altered (Nadel et al 1974) (From IX)

A number of physical and chemical processes in the body are thermosensitive. Thus the blood viscosity increases as its temperature drops and the rate of diffusion and enzyme activity declines. When the temperature of the blood drops a smaller volume of oxygen is given off from the hemoglobin at a given oxygen pressure (the Bohr effect). In maximal work, however, oxygen extraction locally in working muscles should be almost maximal, thereby independent of variations in the oxygen binding capacity of hemoglobin due to small changes in blood temperature. In conclusion, the factors mentioned may aggravate the distribution of oxygen to working muscles and the mitochondrial oxidation.

Local factors

Limiting factors in the oxygen transport chain are also linked to the gas exchange between capillaries and muscle cells and to properties of the muscle cell and its environment. Gollnick et al (1972) found in five elite swimmers a high percentage of low twitch fibers in shoulder muscles (average 75%) whereas the proportion of high twitch fibers was equal to or somewhat less than the value found in athletes. In other endurance sports (average 60%). Myoglobin is suggested to facilitate oxygen utilization by enhancing oxygen transport through the cytoplasm. Muscle myoglobin content is increased (Whipple 1926; Pottenger & H. Hoosy 1967) and

Fig 22 Change in T_{es} after 20 min of submaximal swimming in water at three different temperatures (left) In the right panel this change in T_{es} is related to subject skinfold thickness. Regression line drawn for each temperature. (From IX)



Heat production certainly increases in conjunction with muscular activity e.g. as in shivering or when exercising. The low degree of efficiency in swimming (see the following section) means that 90-95% of the energy output degenerates into heat. However, both shivering and swimming increase the tissue conductance (Burton & Bazett 1936, Edwards 1950) leading in cold water to the emission of more heat than is produced during work. Therefore the body is cooled more rapidly than would be the case if the body were kept inactive (Keatinge 1961).

In water at 18°C and 26°C no accelerated cooling was noted in our subjects (VIII, IX). Mean skin conductance was relatively independent of swimming speed

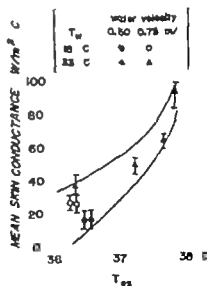


Fig 23 Mean skin conductance in relation to T_{es} during swimming in water at two different water temperatures. Mean values for three subjects. (From VIII)

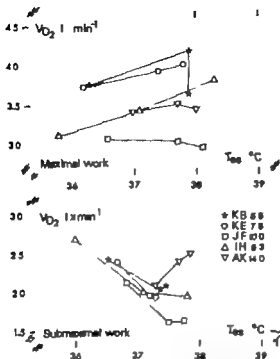


Fig 24 Oxygen uptake measured during submaximal (lower panel) and maximal swimming (upper panel) in relation to T_{re} . Values for subject skinfold thickness are entered (From IX)

suggesting that in this temperature range elevated heat production caused by work was beneficial to reduce the rate of body cooling. In warm water skin conductance increased sharply as swimming speed increased thereby preventing rapid storage of heat in the body (Fig. 23).

With a low internal body temperature ($T_{re} < 37^{\circ}C$) oxygen peak at a given submaximal speed was raised by about $5 \text{ l} \cdot min^{-1}$ and the highest oxygen peak which could be achieved by thin subjects was 6–18% less than at a normal or raised T_{re} (Fig. 24). It should be emphasized that the elevated oxygen uptake in submaximal swimming plus the reduced maximal oxygen uptake severely restrict swimmer performance and reduce endurance.

To summarize persons with a thick layer of subcutaneous fat are better equipped than thin persons to withstand cooling in cold water. The rate of cooling can be influenced to some extent by increased heat production through voluntary exercise or shivering and reduced peripheral circulation. In water at 18–26 $^{\circ}C$ the benefit of

increased heat production during exercise in most people outweighs the liability of the increase in convective heat loss achieved in conjunction with vigorous swimming that accompanies the increased heat loss (cf Craig & Dvorak 1968). However, our data suggest that very thin persons may be exposed to an accelerated cooling even in water at 18–20°C in conjunction with increased work. Rest in still water results in a lower level of heat production but also in lower values for skin conductance than in swimming and may therefore be an equally good means of avoiding severe cooling. It should be noted that clothing reduces skin to water heat loss (Keatinge 1961).

Energy output and efficiency of different styles and strokes

The results in this section are primarily based on studies I, II, III, VI and VII.

The efficiency of propulsion in water depends to a great extent on the swimming style. This is readily apparent on a glance at existing world records (Table 3).

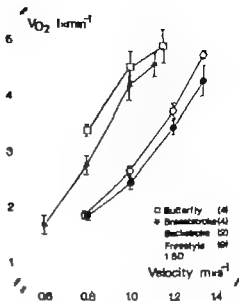
Table 3 World records for men swimming 200 m (1973). Mean swimming velocity and the percentage of freestyle swimming speed are shown.

	Time min	Velocity m s ⁻¹	%
Breaststroke	2 19.28	1.44	81
Freestyle	1 52.80	1.77	100
Backstroke	2 01.87	1.64	93
Butt. fly	2 00.70	1.66	94

Fig. 25 shows mean values for oxygen uptake for elite swimmers swimming different styles. Oxygen uptake was 1.2 l min⁻¹ less in the freestyle and backstroke than in the breaststroke and butterfly. The maximal swimming speed in the freestyle and backstroke was about 0.25 m s⁻¹ faster than in the other two swimming styles. Mean maximal oxygen uptake by elite swimmers amounted to 4.6–4.9 l min⁻¹ in the backstroke, breaststroke and butterfly (Fig. 25). The somewhat lower maximal oxygen uptake in the freestyle in this study (4.3 l min⁻¹) can be explained by the fact that this section also comprised a few less well trained swimmers.

The results shown in Fig. 25 are in good agreement with world records with the exception of the butterfly. It should be noted that the work durations for existing world records were about 2 min, whereas the swimming reported in Fig. 25 lasted 5–6 min. In two minutes, maximal work capability is governed by anaerobic

Fig 25 Mean values for oxygen uptake in relation to swimming speed by male elite swimmers in the four competitive swimming styles. Number of swimmers is denoted within parenthesis. Oxygen uptake levelled off when speed was further increased (not included in the graph) (From VII)



power to a greater extent than in 5-6 min of work in which maximal aerobic power is a limiting factor.

The breaststroke and butterfly provide somewhat unfavourable mechanical conditions for propulsion in water. In the breaststroke both the arms and legs are recovered in the water, leading to high drag values. Arm recovery in the butterfly makes it necessary to lift part of the upper body out of the water. This lift probably consumes a great deal of energy and makes it impossible to maintain a high speed for any great length of time.

Oxygen uptake in the freestyle and backstroke appears to display an exponential increase as swimming speed increases, whereas the increase of oxygen uptake during the breaststroke and butterfly was linear or somewhat less at high speed (Fig. 25). This may be because the swimming strokes in both these styles are symmetrical, resulting in great deceleration in propulsive phase, so as to compensate for the retardation in non-propulsive phase, than is the case in the more continuous propulsion in the freestyle and backstroke. Therefore efficiency probably increases when the ratio of swimming stroke increases at higher speed.

Efficiency in swimming was poor (4.7%) as compared to cycling (20-22%) but of the same magnitude as reported by di Prampero et al. (1974) using a similar measuring technique. The freestyle displayed greater efficiency (6.7%) than the breaststroke (4.6%). An untrained swimmer displayed an efficiency of 2.7% in the breaststroke (VI).

The greatest efficiency (7.7%) was measured in the freestyle using only the arms.

whereas leg kicks in both the breaststroke and the freestyle displayed poor efficiency (2-4% and 1-2% respectively) (VI)

From the physiological point of view maximal performance in swimming is mainly governed by two factors viz. the maximal power of energy yielding processes and the degree of efficiency (swimming technique)

Elite swimmers especially in distance events are distinguished by high maximal aerobic power (I-III-VII). Maximal oxygen uptake for twelve male swimmers was found to be 5.05 l min^{-1} (range $4.04-5.93$) in swimming and 5.38 l min^{-1} ($4.73-6.42$) in running. The corresponding values for female swimmers were 3.42 l min^{-1} ($2.94-3.73$) and 3.64 l min^{-1} ($3.35-4.04$) respectively. In relation to body weight maximal oxygen uptake in running amounted to $68.6 \text{ ml kg}^{-1} \text{ min}^{-1}$ ($62.5-76.4$) for male swimmers and $55.3 \text{ ml kg}^{-1} \text{ min}^{-1}$ ($47.6-61.2$) for the female swimmers (III).

OXYGEN UPTAKE liters/min

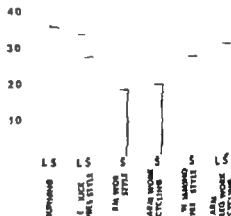


Fig. 26 Oxygen uptake during various types of exercise with maximal intensities for 3-5 min. Open symbol refers to the untrained (S) and closed symbols to the elite swimmer (L).

These values were comparable to those obtained in other endurance events (Saltin & Astrand 1967; Ekblom & Hermansen 1968). Swimmers generally weigh more than e.g. runners, which is why their maximal oxygen uptake values expressed in $\text{ml min}^{-1} \text{ kg}^{-1}$ are lower than for good runners. In water body weight is by no means the same loading factor as in running (I-IV). This suggests that a swimmer's maximal oxygen uptake should be expressed in l min^{-1} .

Oxygen uptake during maximal running uphill may be regarded as an absolute measure of an individual's maximal aerobic power. Elite swimmers as already mentioned displayed a 6-7% lower oxygen uptake in maximal swimming. Maximal oxygen uptake in untrained swimmers was reduced by about 20% in swimming as compared to running (I). This training enhances physical performance, but it is important for this training to take place in water to the greatest possible extent.

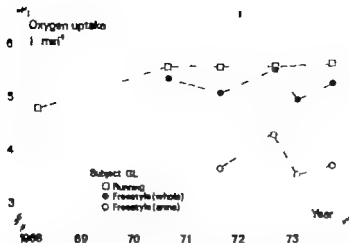


Fig 27 Oxygen uptake during swimming and running at maximal intensities by one world-class swimmer over the past six years. Note the unchanged oxygen uptake during running from 1970 where oxygen uptake during swimming with the whole stroke and with only the arms varied considerably during the seasons. (From Holmér 1973.)

Two sisters (identical twins) displayed the same maximal oxygen uptake in running but the one who participated in hard swim training displayed a 24-49% higher oxygen uptake in different swimming styles than her sister who was not specially trained by swimming (Fig 26). (It should be emphasized that both of the girls were taking part in other intensive physical training.) Results from study II (Fig 26) therefore suggest that it is swim training which produces the main performance improvement in swimming.

Fig 27 shows oxygen uptake in maximal swimming and maximal running by a world-class swimmer during several years of intensive swim training. Varying oxygen uptake values were measured during maximal swimming owing to variations in training intensity, illness etc. Peak values were noted during periods in which the swimmer was most successful. The variation in oxygen uptake was most pronounced in freestyle swimming using only the arms (as mentioned earlier, most important to propulsion in the freestyle). It should be emphasized that running training was only performed to a very limited extent. Oxygen uptake in maximal running was still consistently higher than or equal to oxygen uptake in maximal swimming.

A poor swimming technique by definition explains why untrained swimmers at given speed required a higher oxygen uptake than trained swimmers. (1) A distinguishing feature of swimming is that individual differences in technique (efficiency) are more important to performance than in the case of cycling (Åstrand 1952, Åstrand

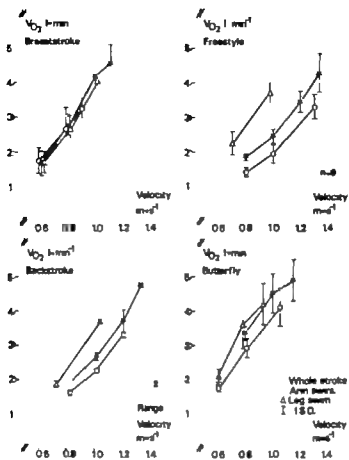


Fig. 28. Mean values for oxygen uptake during swimming with arm strokes, leg kicks and the stroke as a whole in relation to velocity. (From VII)

.960 Hermansen & Saltin 1969) and running (Margaria et al. 1963, Hermansen & Saltin 1969) and that efficiency increases markedly with training (VI)

Oxygen uptake in the breaststroke at a given submaximal speed was the same irrespective of whether the swimmer used the entire swimming style or arm strokes only or leg kicks (Fig. 28). From the propulsion point of view, arm strokes were most important in the other three swimming styles, whereas leg kicks, especially in the freestyle, were less economical (Fig. 28). It should be noted in this respect that during swimming with arm strokes alone, the body probably attained a more favourable position in the water, with a cork plate between the swimmer's thighs than without a support (Fig. 5, p. 19). However, the body position should be similar to the one in swimming with the entire stroke when leg action exerts a lift on the lower part of the body. The highest oxygen uptake which could be produced with

arm strokes only or leg kicks only amounted to 71-83% and 79-90% respectively of highest oxygen uptake for the entire swimming style. Maximal swimming speed with arm strokes alone was less than with the entire swimming style owing to the clearly lower oxygen uptake but only slightly lower in the freestyle due to the high efficiency of arm strokes here (VI).

Thus there were variations in efficiency both among the different swimming styles and even within each swimming style with respect to arm stroke and leg kick efficiency. It is therefore important for the swimmer to give priority to the movements which provide the greatest propulsive efficiency especially when energy supply may be a limiting factor. In this context it should be kept in mind that the highest energy output which can be produced with the different types of swimming strokes does vary and therefore may have an effect on endurance in the different swimming styles.

Concluding remarks

The load on the skeleton joints and ligaments is slight in water owing to the buoyancy. For most people treading water and swimming at a modest speed impose a load on the oxygen transport organs whose magnitude is equal to or greater than in fast walking. These circumstances make swimming a suitable form of physical training especially in previously untrained sedentary and older subjects. In these categories of people training by running sometime implicates troublesome complications from joints tendons and muscles of the lower extremities (Nilsson et al. 1989). Some caution may be advised for people with heart trouble as swimming leads to a greater rise in blood pressure than does running.

One practical consequence of the very large variations in energy metabolism noted in different people swimming at a given speed and using different swimming style is worthy of note. A top swimmer swam the freestyle at a speed of 0.6 m s⁻¹ with an energy output of 560 W whereas another well-trained person (a trained swimmer) needed all of 1100 W (Fig 5). Total energy consumption over a given period of time may vary strikingly depending on the swimming style used (Fig 25). The popular statement that swimming for 10 min consumes 400 kJ is an over simplification as it is also the case in comparison of the energy yielded by various work forms.

A few conclusions of practical significance should be further underlined. Maximal oxygen uptake during running can not be employed to predict performance in swimming. It is obvious that a relatively large change in oxygen uptake may take place during maximal swimming without affecting maximal oxygen uptake during running and that intensive swim training need not be linked to any increase in maximal oxygen uptake during running.

When heart rate is used as an indicator of the load on oxygen transporting organs during training it should be remembered that this variable is clearly lower in maximal swimming than in maximal running and cycling

At Swedish latitudes in which outdoor water temperatures seldom exceed 20°C a long stay in cold water presents an obvious risk which is especially pronounced in thin people and small children. The great heat conductance of water may produce a drop in body temperature of several degrees within half an hour. This risk factor must therefore be kept in mind and measures taken to counteract the cooling of the body during activities in cold water.

SUMMARY

1. Man's physiological responses to swimming were studied in 87 subjects of varying skill and training status in swimming. A swimming flume provided excellent facilities for more detailed analyses of circulatory and respiratory responses as well as energy exchanges of swimming man during more controlled conditions than was possible in previous investigations. Running and cycling tests were also performed for purposes of comparison.
2. The main loading factor during water treading and swimming at low speed was the body's sinking force (W_F) (15.47 kp for female and 49.56 kp for male subjects). Oxygen uptake during water treading was given by the equation $\dot{V}O_2 = 0.33 \times W_F$ ($r = 0.96$). The sinking force displayed a strong correlation with different anthropometric variables e.g. fat-free soft tissue ($r = 0.90$) and fat weight ($r = 0.76$).
3. At moderate and higher swimming speeds the drag on the body increases exponentially and constitutes the main loading factor. Oxygen uptake during swimming at a given submaximal speed was dependent on degree of swimming training, body dimensions, swimming technique and swimming style. The oxygen uptake at a given submaximal speed was generally higher for untrained subjects and tall subjects compared with trained swimmers and small subjects respectively. The increase in oxygen uptake with increasing swimming speed was linear or slightly exponential. Maximal oxygen uptake in swimming was for elite swimmers 5-7% lower than in running and approximately the same in cycling. Corresponding values for untrained swimmers were 20% and 10% lower respectively.
4. When the body was submerged vital capacity was 10% reduced and the expiratory reserve volume was less than 1 liter compared with 2.5 liters in air. The increase in tidal volume was affected in water essentially by the size of the inspiratory reserve volume. At corresponding submaximal $\dot{V}O_2$ level \dot{V}_E and $V_E \dot{V}O_2$ were approximately the same in swimming and running. During maximal swimming mean values for V_E and $V_E \dot{V}O_2$ were significantly lower than in maximal running or cycling. Alveolar ventilation per breath was greater during both submaximal and maximal swimming as compared with running. Values for arterial O_2 saturation, O_2 and CO_2 content and O_2 and CO_2 pressure during both submaximal and maximal work were the same in swimming and running.
5. Heart rate, cardiac output and stroke volume during submaximal swimming were of the same magnitude and increased with increasing speed in approximately the same way as during running. Heart rate was significantly lower (12-15 beats

min^{-1}) in maximal swimming than in maximal running. Stroke volume was the same while cardiac output was lower in maximal swimming compared with maximal running. Maximal $\alpha\text{-}\bar{v}\text{O}_2$ difference in swimming was 14.8 vol% and in running 16.0 vol%. In exercising leg the $\alpha\text{-}\bar{v}\text{O}_2$ difference was 17.5 vol% and 17.7 vol% in maximal swimming and running respectively.

6. In cold water (18°C) thin subjects suffered a $0.2\text{--}1.6^\circ\text{C}$ fall in T_{es} in conjunction with 20 min of submaximal swimming. In water at 18°C and 26°C changes in T_{es} were related to individual values for skinfold thickness. The convective heat transfer coefficient for the heat flow from skin to water was in still water $230\text{ W m}^{-2} ^\circ\text{C}^{-1}$ and in flowing water $460\text{ W m}^{-2} ^\circ\text{C}^{-1}$. The value for the coefficient during swimming was $580\text{ W m}^{-2} ^\circ\text{C}^{-1}$ and it was independent of swimming speed. $T_{\text{sk}} - T_{\text{w}}$ was $1\text{--}2^\circ\text{C}$ at rest in still as well as in flowing water and always less than 1°C during swimming. $T_{\text{m}} - T_{\text{es}}$ was always positive, the difference being smallest in cold water (18°C). Mean skin conductance during swimming in water at 18°C was relatively independent of speed and approximately $20\text{ W m}^{-2} ^\circ\text{C}^{-1}$ while in water at 34°C mean skin conductance increased exponentially with T_{es} and averaged $100\text{ W m}^{-2} ^\circ\text{C}^{-1}$ at 0.75 m s^{-1} and $T_{\text{es}} = 37.8^\circ\text{C}$.
7. With lowered internal temperatures ($T_{\text{es}} < 37^\circ\text{C}$) shivering occurred in subjects both at rest and during swimming. Thus oxygen uptake was elevated by approximately 0.5 l min^{-1} at a given swimming speed. In maximal swimming oxygen uptake was 6–18% reduced for subjects with $T_{\text{es}} < 37^\circ\text{C}$ than at normal or raised T_{es} . It was concluded that the elevated oxygen uptake in submaximal swimming plus the reduced maximal oxygen uptake severely restrict a swimmer's performance and reduce his endurance.
8. Breaststroke and butterfly swimming demanded 1.2 l min^{-1} higher oxygen uptake at a given submaximal speed than did freestyle and backstroke. Mechanical efficiency in swimming was low: 4–6% in breaststroke and 6–7% in freestyle by elite swimmers. When using only the arms in freestyle swimming efficiency was 7–7% while the leg kicks displayed poor efficiency (2–4% in breaststroke and 1% in freestyle).
9. Maximal oxygen uptake for male elite swimmers was 5.1 l min^{-1} in swimming and 5.4 l min^{-1} in running. Corresponding values for female swimmers were 3.4 and 3.6 l min^{-1} . In relation to body weight maximal oxygen uptake in running amounted to 69 and $5\text{ ml min}^{-1} \text{ kg}^{-1}$ for male and female swimmers respectively. Maximal oxygen uptake in swimming varied as a consequence of swimming training while during the same period of time maximal oxygen uptake in running remained relatively unchanged. In other words maximal oxygen uptake in running

is not representative for performance in swimming and training of a swimmer's maximal oxygen uptake should to the greatest possible extent take place by swimming

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ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 408

**ADAPTIVE CHANGES OF CARDIOVASCULAR DESIGN IN
SPONTANEOUS AND RENAL HYPERTENSION**

Hemodynamic studies in rats.

BY
YEN LUNDGREN

GÖTEBORG 1974

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This summary is based on studies reported in the following papers:

- I Structurally based increase of flow resistance in spontaneously hypertensive rats
B Folkow M Hallböök Y Lundgren and L Weiss Acta physiol scand 1970 79 373-378
- II Renal vascular resistance in spontaneously hypertensive rats
B Folkow M Hallböök Y Lundgren and L Weiss Acta physiol scand 1971 83 96-105
- III Rate and extent of adaptive cardiovascular changes in rats during experimental renal hypertension
Y Lundgren M Hallböök L Weiss and B Folkow Acta physiol scand 1974 In press
- IV Regression of structural cardiovascular changes after reversal of experimental renal hypertension in rats
Y Lundgren Acta physiol scand 1974 In press
- V Blood pressure and vascular design in renal hypertension in rats after prolonged propranolol treatment
Y Lundgren Acta physiol scand 1974 In press

The papers are referred to in the text by their Roman numerals

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INTRODUCTION

Increased activity of the precapillary smooth muscles has for decades been considered to be the background of the increased resistance to flow in established hypertension. Very little attention has been paid to the possible hemodynamic consequences of the media hypertrophy in arteries and precapillary resistance vessels despite the fact that such changes in vascular design have been recognized by morphologists for over 100 years. Recently quantitative hemodynamic analyses initially in man (see Folkow 1956, Sivertsson 1970) and later in spontaneously hypertensive rats (e.g. Folkow et al 1970, 1973) have shown that structural changes of the resistance vessels are indeed of great hemodynamic importance. Thus the mentioned studies revealed that the resistance vessels display an increased resistance even during maximal dilatation and exaggerated resistance increases upon smooth muscle contractions implying that resistance will be increased even when smooth muscle tone is normal. This effect of a changed vascular design may be denoted structural autoregulation as a structural correlate to the functional autoregulation characterizing precapillary vessel responses upon acute changes in pressure.

The present series of investigations based on five individual studies that are below denoted I - V was performed in order to add further knowledge to the background of the increased resistance to flow both in primary hypertension and for comparison in the most common type of secondary hypertension i.e. renal hypertension. For such purposes the adaptive structural changes in cardiovascular design were studied in more detail in the spontaneously hypertensive rat (SHR; Okamoto 1969) which is probably the best animal model so far for essential hypertension in man and in the renal hypertensive rat (RHR). In SHR studies utilizing quantitative perfusion analyses were performed concerning resistance vessel design of the entire systemic vascular bed and of the renal vascular circuit. Furthermore the extent and time course of the development and regression of cardiovascular hypertrophy were studied utilizing the renal hypertensive rat. Finally since preventive propranolol treatment seems to exert a protective influence in SHR against the progress of hypertension and hypertrophic vascular changes (Folkow, Lundgren and Weiss 1972) it was explored how the same type of preventive treatment with β -receptor antagonists affected the development of renal hypertension and the associated hypertensive vascular changes.

PREVIOUS STUDIES

History

Hypertensive disease was known to exist for a long time before the technique of indirect pressure measurement was developed during the latter half of the nineteenth century. Therefore characterization of the disease was initially based mainly on the morphological changes of heart arteries and kidneys after death. Bright (1836) described a series of cases with albuminuria in combination with three different stages of degenerative kidney and found a marked hypertrophy of the left ventricle. In a large number of these cases he did not find any organic cause for the cardiac hypertrophy and suggested that an altered quality of the blood might either have affected the heart directly or that it so affected the vessels that an enhanced cardiac force was needed.

Later Gull and Sutton (1872) concluded that the arterioles and the capillaries were altered throughout the body in chronic Bright's disease due to a hyaline fibroid formation in the arteriolar walls and a hyaline granular change in the capillaries. These morphological changes in the arterioles and capillaries were thought to be the primary and causative change in chronic Bright's disease with contracted kidney. However Ewald (1877) confirming Johnson's (1868) findings that the media hypertrophy was associated with cardiac hypertrophy considered the vascular changes to be a consequence rather than the cause of hypertension.

Mahomed (1879-1881) who studied chronic Bright's disease found that the cardiovascular changes often preceded the renal changes and concluded: "What has been cause in one case may be the result in this general disorder may cause high arterial pressure and this in its turn kidney changes while on the other hand kidney changes may be primary and acute and they may in their turn produce impurity of blood and this general high pressure. But whether we read the tale backwards or forwards it is the same tale in the end."

A hypertensive disorder with quite a different course than Bright's disease of the kidneys was first recognized by Muchard (1889), von Bosch (1893) and Albutt (1915). The term essential hypertension was first used by Frank (1911) and the modern classification of hypertension is based on a combined clinical and pathological study by Volhart and Fahr (1914). However since the etiology of essential hypertension has remained unknown the question as to whether genetic factors were involved arose early. In 1923 Weltz noted a more frequent occurrence of high blood pressure in brothers and sisters of

patients with essential hypertension than in control subjects

The earlier discovered media hypertrophy was confirmed by Turnbull (1915) who stated that it was constantly associated with high blood pressure and well correlated to the degree of the cardiac hypertrophy. Furthermore Kernohan, Anderson and Keith (1929) found that media hypertrophy was the major component of the increased thickness of the arterial wall relative to lumen.

To study hypertensive disease in more detail different kinds of experimental hypertension were introduced. The most common methods used were renal artery constriction (Goldblatt et al 1934, Drury 1938, Pickering and Prinzmetal 1938) and later the administration of deoxycorticosterone acetate (DOCA) (e.g. Selye, Hall and Rowley 1943). Generalized media hypertrophy in medium sized and small arteries was observed in dogs with experimental renal hypertension (Goldblatt 1938 and Child 1938). The recent introduction of animal models with primary hypertension which more closely resemble the most important type of hypertension in man, essential hypertension, provides a valuable experimental tool in the study of hypertensive disease.

Background of increased arterial pressure

According to Poiseuille's law and Frank's formula mean arterial pressure is determined by the relationship between cardiac output and peripheral resistance. Thus an increased arterial pressure may be due to an increased cardiac output and/or to an increased peripheral resistance. An increased peripheral resistance may in turn be due to a raised viscosity of the blood, an increased length of the vessels and/or to a reduced cross-sectional area of the vascular bed.

Cardiac output

An increased cardiac output has been observed in early phases of essential hypertension by e.g. Werko and Lagerlöf (1949), Sannerstedt (1966), Lund-Johansen (1967), Julius and Conway (1968), Frohlich et al (1970) and Julius and Schork (1971). However in established essential hypertension cardiac output usually seems to be within normal limits during rest (e.g. Goldring and Chasis 1944, Freis 1960, Bello Sevy and Harokai 1963, Frohlich et al 1970). In renal hypertension a transient early increase in cardiac output has often been noted (e.g. Ledingham and Cohen 1964, Ferrario, Page and McCubbin 1970, Bianchi et al 1972) but also in this type of hypertension the increased resistance seems to dominate later phases. Thus in both primary and

secondary (renal) hypertension the increased resistance is the dominating feature in the established phase

Peripheral resistance

According to Poiseuille's law resistance to flow is determined by viscosity, vascular length and internal radius. Blood viscosity seems to be normal in established essential hypertension (Pickering 1968). In young hypertensive subjects blood viscosity as measured *in vitro* was found by Tibblin et al (1966) to be 20 per cent higher than in normotensives. Only slight changes in hematocrit and plasma viscosity were noted. However, *in vitro* measurements of blood viscosity must be judged with caution since they are often unreliable indicators of the viscosity *in vivo* (Djolosugito et al 1970). In renal hypertension changes in hematocrit have not been found (e.g. Ledingham and Pelling 1967, Ferrario, Page and McCubbin 1970). No studies provide evidence that changes in the length of resistance vessels are of such magnitude to be of any relevance for the increased resistance to flow. It is therefore generally agreed that the increased resistance in hypertension is primarily due to a reduction in average internal radius of the resistance vessels and it has as mentioned above been more or less generally assumed that this should be due to an increased vascular smooth muscle activity. Such an increased smooth muscle activity could then in turn be due to a greater sympathetic activity, increased amounts of humoral vasoconstrictor agents, increased smooth muscle sensitivity or "reactivity" to constrictor influences, changes in the electrolyte balance and/or to changes in basal myogenic tone.

Sympathetic tone: There is no clear evidence of any constantly increased sympathetic tone at rest either in established primary hypertension (see Pickering 1968, Wallin, Dellus and Hogbarth 1973) or in secondary renal hypertension (cf. Page and McCubbin 1968). This by no means denies that sympathetic influences are of great importance but act in a different manner as will be discussed later.

Humoral constrictor agents: Increased amounts of circulating constrictor agents have not been found in primary hypertension at least not in concentrations high enough as to alone explain the increased resistance level (cf. Pickering 1968, Okamoto 1972). The importance of the renin-angiotensin system and its interaction with the sympathetic nervous system, particularly in renal hypertension, has been much debated but is still not exactly understood (cf. Page and McCubbin 1968, Joy 1971).

Vascular reactivity: Concerning increased smooth muscle sensitivity or

*reactivity to constrictor agents this possibility has been extensively studied in a great number of studies of blood pressure changes and changes in total or regional resistance to constrictor agents (cf. Freis 1960, Page and McCubbin 1965, Doyle 1968, Lavery, McGregor and McQueen 1968, Page and McCubbin 1968, Pickering 1968, Sivertsson 1970, Mandlowitz 1973). However, the results are often contradictory and sometimes even confusing, since both experimental approach and definitions differ considerably. It should be realized that increased blood pressure responses and increased vascular responses to injected constrictor agents can have widely different backgrounds and that furthermore, increased vascular reactivity does not necessitate any increased smooth muscle reactivity or sensitivity. In case structural increases in wall/lumen ratio are also present (see also Sivertsson 1970, Tobian 1972, Johansson 1974).

Electrolytes: It has been suggested that changes in electrolyte composition might lead to increased smooth muscle activity (Friedman, Friedman and Nakashima 1957, Pickering 1968). According to Freis (1960) there is no evidence of any decisive changes in electrolyte composition leading to increased vascular smooth muscle tone in established uncomplicated primary hypertension. However, changes in the ion composition have been found in spontaneously hypertensive rats (Nagaoka, Kikuchi and Aramaki 1970, Jones 1973) but further studies are needed to settle this question. Considering end hypertension alterations in renin-angiotensin-aldosterone secretions may influence the electrolyte balance in such a way as to increase smooth muscle tone but such mechanisms have not so far been shown to be of major importance (cf. Page and McCubbin 1968).

Basal myogenic tones: Hardly any studies are available that more directly deal with the possibility of an increased myogenic tone as the background of the raised resistance (cf. Freis 1960).

Structural changes in resistance vessel design: The increased flow resistance found in hypertensive subjects may be due to mechanisms quite different in nature from those described above. Thus, instead of a functional luminal narrowing it is a priori quite possible that a structural luminal reduction exists as a result of an increased wall thickness. For example, Tobian and Binlon (1952) (cf. Tobian 1972) suggested that an increased amount of water and electrolytes in the arteriolar walls i.e. water logging might exist causing an increased wall thickness with a consequent luminal narrowing. This hypothesis was mainly based on analyses of the water and electrolyte contents in conduit arteries from patients who died in hypertensive disease. How-

secondary (renal) hypertension the increased resistance is the dominating feature in the established phase

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Vascular reactivity: Concerning increased smooth muscle sensitivity or

has been most convincingly demonstrated with different morphological and histochemical techniques but it is exceedingly difficult with such techniques alone to estimate the hemodynamic consequences which in the final end is the crucial point

HEMODYNAMIC CONSEQUENCES OF MORPHOLOGICAL VASCULAR CHANGES

Theoretical considerations

Flow resistance (R) is commonly used as an index of the average internal radius (r_i) of the resistance vessels in hypertension since factors like vascular length and blood viscosity appear to be largely unchanged, as mentioned above. Since R is inversely proportional to the fourth power of r_i , any change of r_i becomes greatly amplified when expressed as R . The resistance at complete vascular smooth muscle relaxation (R_{min}) would then reflect the structurally determined r_i and in a way be an expression of the resting length of the encircling muscle elements. Under standardized conditions R_{min} would thus, in a way imply a structurally defined baseline from which current levels of vascular smooth muscle activity can be judged as the ratio between the prevailing R level and R_{min} .

However, when a wall thickening becomes involved it will amplify the luminal reduction induced by a given smooth muscle shortening thereby distorting the relationship between R and R_{min} . Therefore the entire dynamics of resistance vessels will become markedly changed whenever their structurally determined wall/lumen ratio is altered (cf Folkow et al 1973). Concerning adrenergic excitatory influences e.g. those exerted by the vasoconstrictor fibres, the smooth muscle contraction of the resistance vessels is initiated from the outer muscle sheath since all the neuroeffector functions are placed at its adventitial border (cf Ljung 1970). The thicker the media (and other wall layers placed inside the media), the more pronounced the luminal reduction for any given level of smooth muscle shortening since the wall mass is constant. Thus a vascular hyperreactivity is created whenever the wall/lumen ratio is increased which does not call for any smooth muscle hyperreactivity or hypersensitivity. It also follows that the resistance becomes higher for any given level of smooth muscle tone. Furthermore, in case the lumen is reduced even at complete smooth muscle relaxation, the very baseline for vascular responses is raised which will increase resistance even more for any given degree of smooth muscle shortening. However, even in case R_{min} is the same or even decreased, an enhanced wall/lumen ratio will create a vascular hyperreactivity of great importance for resistance control.

Such changes in vascular design would thus imply a structural autoregulation of the resistance vessels enabling them to reset the resistance equilibrium and the range of dilatation-constriction at a higher level without necessitating any increased vascular smooth

muscle tone. Further the thicker walls would make the vessels stiff and the reduced luminal size would balance off the rise in wall tension that would otherwise occur as a result of the raised pressure.

With respect to larger arteries the increased stiffness would help to explain the resetting of the baroreceptors (Aars 1969) and the related hypertrophy of the left ventricle will obviously imply a suitable compensation for the imposed work load on the left heart. Thus changes of this general nature can be expected to have far-reaching hemodynamic consequences when once established in hypertension.

Ex vivo hemodynamic studies of the resistance vessels

Hemodynamic studies on regional vascular beds in man (Folkow 1956, Folkow, Limby and Thulesius 1958, Conway 1963) suggest that the raised flow resistance in established hypertension may predominantly be due to a structural change of the resistance vessels. Thus even during complete vascular smooth muscle relaxation resistance to flow in the forearm was increased almost to the same extent as the resting blood pressure. Moreover comparative hemodynamic studies on the hand vascular bed in hypertensive and normotensive subjects revealed not only an increased resistance at maximal dilatation but also exaggerated increases in resistance responses to noradrenaline in the hypertensive subjects though smooth muscle sensitivity to noradrenaline appeared to be largely unchanged (Sivertsen 1970). Thus the resistance vessels of the hypertensive subjects differed from those of normotensive controls first by exhibiting a raised flow resistance even at complete vascular smooth muscle relaxation, second by showing no signs of any enhanced smooth muscle activity in the resting equilibrium since the ratio between resting R and R_{min} was not increased and third by displaying a vascular "hyperreactivity" without any clear evidence of enhanced smooth muscle sensitivity. These findings strongly suggest the presence of a structural change of the regional resistance vessels so organized that they exhibit an increased wall thickness in combination with a decreased luminal size even at maximal dilatation.

To allow more detailed hemodynamic studies of resistance vessel design in primary hypertension the spontaneously hypertensive rat (SHR; Okamoto and Aoki 1963) has been used. SHR which seem to be the best animal model so far for essential hypertension in man have been developed from normal Wistar rats by inbreeding of animals with higher than average blood pressures. Further these rats appear to have a multigenic background of their hypertension as seems to be the case also in human essential hypertension (cf. Pickering 1968).

COMPLEX EXPERIMENTAL RESULTS

PERIPHERAL PRESSURE, mm Hg
PROPORTIONAL TO FLOW RESISTANCE

HYPOTHETICAL RESISTANCE VESSELS

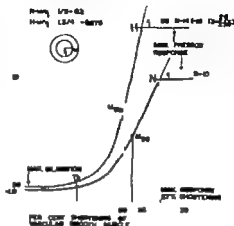
R-RESISTANCE PROPORTIONAL
TO PERIPHERAL PRESSURER=100
L=100
W=100

Fig 1 Left part: Average resistance curves for spontaneously hypertensive rats (SHR) and normotensive control rats (NCR) based on the results of 15 paired experiments. Right part: Mathematically deduced resistance curves for two hypothetical resistance vessels H and N where H differs from N only in the respect that its media thickness is supposed to be increased 30 per cent encroaching on its lumen even at complete smooth muscle relaxation. w/r = ratios of wall thickness to internal radius. Note the striking similarities between the relationships of the two sets of resistance curves with respect to: 1 Resistance at maximal dilatation 2 Threshold (T_h) dose of noradrenaline 3 Steepness of the curves 4 M_{50} i.e. 50 per cent of the maximal pressor response to noradrenaline and 5 Maximal pressor response (Folkow et al 1970)

In a series of experiments the isolated hindquarters of one SHR and one matched normotensive control rat (NCR) were perfused in parallel and their hemodynamic characteristics were compared from maximal dilatation up to maximal constriction (Folkow et al 1970). Thus during constant flow conditions noradrenaline (NA) was infused in a stepwise fashion from subthreshold up to supramaximal concentrations

and the resistance responses were recorded giving the mean dose-response curves for SHR and NCR (resistance curves) shown in the left part of Fig 1. When compared to NCR the SHR resistance curve showed the following hemodynamic characteristics:

- 1 A raised flow resistance at maximal dilatation
- 2 An unchanged NA threshold (i.e. the NA concentration producing 25 per cent increase in resistance from the state of maximal dilatation)
- 3 Exaggerated resistance responses to suprathreshold concentrations of NA expressed as an increased steepness of the resistance curve
- 4 A shift towards the left of the resistance response that corresponds to 50 per cent of the maximal pressor response to NA
- 5 A raised maximal pressor response to supramaximal amounts of vasoconstrictor agents i.e. an increased maximal contractile strength

These 5 characteristics together strongly suggest an increased wall/lumen ratio mainly due to an increased media thickness that also implies a somewhat reduced lumen even at maximal dilatation.

However the hemodynamic consequences of such a structural change is best illustrated by exact computations of resistance changes in idealized model vessels as shown in the right part of Fig 1. This figure illustrates mathematically deduced resistance curves for two hypothetical resistance vessels one normotensive N and one hypertensive H. The ratio between wall thickness w and internal radius r_i of the normal resistance vessel N is set at 1:3 at maximal dilatation (Van Citters 1966). The same ratio for the hypertensive vessel H is assumed to be 1:3.47. Hence the two vessels differ only in the respect that H displays a 30 per cent increase of media thickness which encroaches upon the lumen even at complete smooth muscle relaxation. The wall mass is considered to be constant when N and H constrict which is also in agreement with direct measurements on vessels (Boaz 1969). It is further assumed that constriction is initiated from the outermost muscle sheath since most of the α -receptors seem to be located at the adventitial muscle surface (Johansson *et al.* 1970). This would imply that the constriction caused by e.g. exogenous NA displaces inner tissue layers towards the lumen, which then becomes reduced in an exaggerated way and the more so the stronger smooth muscle contraction and the thicker the wall is to start with. Further the NA concentration is given in a log scale on the abscissa of the left part of Fig 1 while the percentage shortening of smooth muscle length is instead used on the abscissa for the

vascular models in the right part. Since log NA concentration is related to decrease of smooth muscle length by the S formed dose-response curves characterizing e.g. contracting vascular smooth muscle strips, the scales of the two abscissae are made as equal as possible in Fig. 1 by utilizing such curves for transformation.

When comparing the mathematically deduced resistance curves for the two model resistance vessels, the relationship between curve H and curve N is in all essential details closely similar to the experimentally observed relationship between the SHR and NCR resistance curves. In fact, the parallels between the mathematically deduced curves and the experimental curves are so close as to suggest that the extent of structural change of the SHR resistance vessels may have been of a similar or slightly greater magnitude than assumed to be present in H.

Considering other types of structural or functional vascular changes proposed to be involved in hypertension, an increased vascular smooth muscle sensitivity is incompatible with all the observed characteristics of the resistance curves. Threshold NA concentration would then be decreased and the resistance curve would simply be displaced to the left without any increase in steepness. Moreover, isolated aortic strips from SHR and NCR show no difference in NA sensitivity (Hallböök, Lundgren and Wels 1971). As to other mechanisms proposed, several of these would have to be combined to fit the experimentally obtained resistance curves and, in addition, any hemodynamic influence of the morphologically well documented media hypertrophy would then have to be disregarded. Thus, a thickening of only non-contractile wall elements or a mere water logging (Tobian and Binton 1952, Tobian 1972) could explain most differences but not the considerably increased maximal contractile strength of the SHR resistance vessels beyond the minor gain that would result from the smaller r_1 according to Laplace's law. Moreover, a rarification of the resistance vessels, a reduction of their size at an unchanged wall/lumen ratio or an increased length could explain the increased resistance at maximal dilatation and the unchanged NA threshold but not the other hemodynamic characteristics of the SHR vessels. In other words, only the presence of an increased media thickness in association with a slightly reduced lumen of the resistance vessels even at maximal dilatation can alone explain all the demonstrated hemodynamic characteristics of the SHR hindquarter vascular bed. Thus, these results from the so far best animal model of essential hypertension are in complete agreement with the findings in man and with the view that the increased vascular reactivity and flow resistance in established primary hypertension is mainly or entirely caused by the mentioned type of

changed structural design i.e. an increased media thickness of the resistance vessels

These results are further supported by the finding that the SHR resistance vessels are also proportionally less distensible than the NCR ones both at maximal dilatation and at different levels of smooth muscle tone (Halbäck, Lundgren and Weiss 1974). In addition recent more detailed analyses of the site and extent of the increased resistance in hypertension show that the hypertrophic vascular changes in SHR are solely confined to the precapillary resistance vessels and closely proportional to the increased blood pressure level while the capillary section is largely unchanged and the postcapillary resistance section if anything slightly wider and somewhat less reactive to NA in SHR (Folkow et al. 1974).

AIM OF THE PRESENT STUDY

The present study was performed in order to investigate more in detail certain aspects of the cardiovascular structural changes outlined above using the spontaneously hypertensive rat (SHR) and the renal hypertensive rat (RHR) as models. Since all similar studies in man were performed on a few regional vascular beds the total systemic vascular bed of SHR was used in the first study (I) to explore whether the previously mentioned structural vascular changes were generalized throughout the systemic circulation and if so whether they were pronounced enough to contribute markedly to the elevated pressure in established primary hypertension.

The kidneys are of particular interest in hypertension and an increased renal resistance to blood flow has been almost uniformly reported in essential hypertension (cf Pickering 1968) though it is not settled to what an extent this is due to structural or functional factors. For such reasons SHR and NCR kidneys were perfused in parallel to find out whether the renal vascular bed displays any structural narrowing in SHR which would then imply a potential Goldblatt mechanism of possible etiological importance (paper II).

Furthermore it was considered of great importance to find out whether the structural cardiovascular adaptation to increased arterial pressure develops rapidly or if it is a fairly late complication. Thus in order to study the extent and exact time course of the mentioned structural changes renal hypertensive rats were used because the onset of this type of hypertension is far more abrupt than in SHR (paper III). Another important question arising is if animals with primary hypertension (SHR) are genetically more prone to develop cardiovascular hypertrophy in response to increased pressure than those with secondary hypertension (RHR). Even a modest quantitative difference in this respect may have considerable hemodynamic consequences for the resistance vessels and their function because of the tendency of functional and structural factors to reinforce each other. By comparisons between SHR and RHR an attempt was made to find out whether they differ quantitatively in terms of extent of structural changes for a given level of pressure increase.

From a therapeutical point of view it is of interest to know to what an extent and how rapidly regression of cardiovascular hypertrophy may be accomplished if pressure is kept lowered. This was studied by analysis of the cardiovascular changes at different intervals after reversal of renal hypertension (paper IV).

Moreover is it possible to prevent the development of renal

hypertension and its vascular changes by propranolol treatment as seems to be the case in SHR? The results may be of importance in evaluating the nature of the functional mechanisms initiating the two types of hypertension since the involvement of neurogenic mechanisms also in renal hypertension has recently been much discussed. This problem was studied in paper V.

METHODOLOGICAL PRINCIPLES

The experimental animal models

Animal models used earlier for comparison with human essential hypertension all suffer from the drawback that they are induced by renal, hormonal or nervous interferences. In genetically normotensive animals. However, as already mentioned, rat strains with spontaneous hypertension of polygenic background, thus simulating the situation in essential hypertension of man, have been developed more recently by Smirk and coworkers (e.g. Smirk and Hall 1958) and by Okamoto and his group (Okamoto and Aoki 1963). The latter strain has been used in this laboratory.

Spontaneously hypertensive rats (SHR)

The SHR colony was produced by selective breeding of normal Wistar rats that showed a higher than average arterial pressure. Successive brother-sister inbreeding of the colony was then performed and in 1969, when the first SHR group arrived in our laboratory, they had reached the twenty-first generation. Brother-sister breeding was not consistently continued in order to avoid disturbing passenger phenomena that tend to occur in too strict inbreeding. Genetical studies suggest a polygenic background with an additive mode of inheritance of a relatively small number of major genetic elements (cf. Okamoto 1972). Studies by Okamoto and coworkers (cf. Okamoto 1972) strongly suggest that the SHR cardiovascular system is exposed to an increased centrally induced neurohormonal discharge which would constitute one of the key predisposing elements. Further, SHR exhibit exaggerated and often prolonged pressure and heart rate responses to alerting stimuli (Folkow, Hallböök and Welss 1973). Also during anesthesia, young SHR display a hyperkinetic hemodynamic pattern (Pfeffer and Frohlich 1973), apparently resembling a mild defence reaction. Thus, the early hypertensive phase in SHR closely resembles the early "borderline" phase of essential hypertension in man (e.g. Brod 1963, Julius Pascual and London 1971). In general, SHR display signs of an intensified activity not only of the sympathico-adrenal system but also of the ACTH, corticoid and TSH-thyroid systems (cf. Okamoto 1969, 1972).

The SHR hypertension develops gradually with increasing age, but already at 6 weeks of age blood pressure is significantly raised compared to normotensive control rats (NCR) of the same age. At the age of 5-7 months, blood pressure has reached the level of 170-180 mm Hg.

although this varies somewhat with the substrain used. Concerning pathological findings in SHR the incidence of myocardial lesions, nephrosclerosis and malignant vascular lesions is very high in more advanced stages and increases remarkably with severe hypertension (> 200 mm Hg) (cf. Okamoto 1972).

In summary SHR seem to be the best animal model so far for essential hypertension in man and seem to exhibit all its known characteristics (Okamoto 1972). Hence SHR of both sexes in the phase of established hypertension (7-8 months of age) were used for the hemodynamic studies exploring the entire systemic vascular bed (study I) and the renal vascular bed (study II).

Renal hypertensive rats (RHR)

In studies III, IV and V renal hypertensive rats were used. Renal hypertension was induced in 6-7 week old normotensive male Wistar rats by placing a standardized silver clip on the left renal artery during ether anesthesia leaving the right kidney intact. Sixtyfive to eighty per cent of the operated rats developed hypertension (≥ 150 mm Hg) within 3 weeks after operation. Thus by this Goldblatt procedure a marked hypertension developed quite rapidly. In contrast to the more gradual onset of primary hypertension in SHR. Therefore these renal hypertensive rats (RHR) offered particularly favourable circumstances for exploring how soon cardiovascular changes are established once a rapid fairly persistent functional pressure rise is provoked. Since these rats were genetically normotensive they were also used for comparison with the primary SHR hypertension for evaluations as to whether SHR might have a genetic predisposition for developing a quantitatively more pronounced media and left ventricular hypertrophy. RHR also offered possibilities to study the rate and extent of regression of the cardiovascular structural changes since an immediate reversal of the RHR hypertension occurred upon removal of the renal artery clip.

Blood pressure and heart rate measurements

In study I and II before the technique for measuring blood pressure directly in the caudal artery during awake conditions was developed arterial pressure was measured via a polyethylene tube in one of the femoral arteries during Nembutal[®] anesthesia (3-4 mg/100 g body weight i.p.). The tube was connected to a Statham pressure transducer and pressure was recorded on a Grass Polygraph model 7. Nembutal[®] anesthesia is known to depress both ventilation and temperature regulation which must of course affect the condition of the experimental

animals. A tracheal cannula was always inserted to permit free air ways before arterial pressure was measured under anesthesia and this procedure was found to usually increase arterial pressure some 20 mm Hg in SHR and NCR. On the other hand control measurements during awake and anesthetized conditions showed that the addition of Nembutal® usually decreased arterial pressure in both SHR and NCR around 20 mm Hg. This means that the pressures actually given in study I and II seem to be roughly the same as those recorded directly during awake resting steady state conditions. In studies III, IV and V arterial pressure was measured in the caudal artery during awake resting conditions which ought to be the best way of measuring the true resting arterial pressure. The procedure was the following: The caudal artery was cannulated during brief ether anesthesia. The animal was then allowed to wake up and the arterial pressure was recorded by a Statham pressure transducer during resting conditions. Control experiments showed that the pressure level remained very constant when repeated recordings were made for several hours after the brief ether anesthesia. Also heart rate could be recorded via the catheter in the tail artery.

General perfusion procedures

The following methods have been used: Study I: Perfusion of the entire systemic vascular bed (except the coronaries) in SHR and NCR. Study II: Paired perfusion of the isolated renal vascular bed in SHR and NCR. Study III, IV and V: Paired perfusion of the isolated hind-quarter vascular bed in SHR and NCR (Fig. 2).

For details concerning the respective procedures see the individual papers. Below some general principles and considerations concerning the perfusion technique and its evaluation will be outlined.

Perfusion medium

By using artificial perfusions for studies of the resistance vessels, neuro-hormonal influences are eliminated, reversible differences in ion composition of the vascular walls are diminished and vasoactive substances are washed out. In all experiments oxygenated Tyrode solution was used with 4 per cent (3 per cent in study II) of Ficoll (a synthetic polymer of sucrose and epichlorohydrin, m.w. around 80 000, AB Pharmacia, Uppsala, Sweden) as colloid substitute. The temperature of the perfusate was kept at 38°C in the perfusate container, being about 35-36°C when reaching the preparation. The osmotic pressure of the perfusate was around 300 mOsm/l and viscosity determined in ■

Wells Brookfield Micro viscosimeter was about 1.0 to 1.2 cP at 37°C depending on whether 3 or 4 per cent Ficoll was used. Further different batches of Ficoll were used in different series of experiments due to a limited supply which slightly affected both viscosity and effective colloid osmotic pressure and hence some of the measured parameters. However since all experiments except those in study I were run as equally treated pairs i.e. one SHR (or RHR) in parallel with one matched NCR the two vascular beds were always exposed to exactly the same perfusate and perfusion conditions largely cancelling out accidental interferences by e.g. differences in viscosity, temperature or composition of the perfusate. On the other hand such interferences probably explain why e.g. the mean values of the various NCR groups in study III, IV and V differ slightly. Such unavoidable though minor differences only serve to illustrate the great advantage of using paired experiments in quantitative comparative studies of this type. Further the maximal pressor responses will to a slight degree be affected by a somewhat varying edema formation (see study III, IV and V) which moderately raises tissue pressure and correspondingly reduces transmural vascular pressure. This factor is of course increasingly important towards the end of the experiments. Since edema formation was usually more pronounced in NCR this source of error tends to slightly over estimate the NCR maximal pressor response compared with that of SHR and RHR.

Pressure-flow relationships during maximal dilatation

The pressure-flow relationships during complete vascular smooth muscle relaxation were examined in all 5 studies. The vascular bed was considered to be maximally dilated when no further relaxation could be obtained and to ensure complete smooth muscle relaxation repeated injections of papaverine were regularly used until no further resistance decrease was obtained.

In order to further secure that the vascular bed was really maximally dilated it was checked that perfusion pressure returned to the same control level after sudden increases of flow rate. No signs whatsoever of autoregulation were observed.

When maximal dilatation was ensured the pressure-flow relationships were examined by changing the flow rate in a random fashion while recording the perfusion pressures. Perfusion pressure was except in occasional tests kept below 40 mm Hg (60 mm Hg in study II) in order to avoid rapid edema formation in these maximally dilated vascular beds. Thereafter pressure-flow curves were constructed for each individual experiment. From these curves mean pressure-flow curves were later deduced.

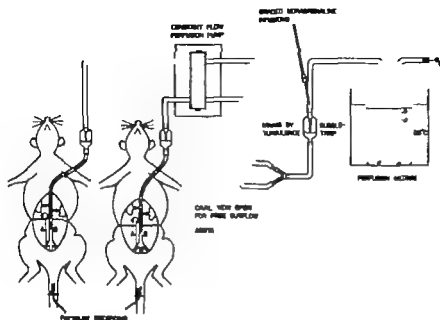


Fig. 2 Paired perfusion of the isolated hindquarters of one renal hypertensive rat (RHR) and one normotensive control rat (NCR) as used in the studies III-V. The perfusate emerges from a common container which is kept at 38°C and drained by a single tube coupled in series with a small mixing chamber that is steadily shaken. The tube emerging from the mixing chamber branches into two tubes which after passing through a double Harvard perfusion pump are connected to the respective aortic cannulas. After passage through the two parallel-coupled rat beds the perfusate is allowed to leave freely via the cut ~~W~~erlor caval veins. Perfusion pressures are measured via the caudal arteries.

Noradrenaline (NA) infusion during constant flow conditions

This procedure was used in study II V and was identical in all studies except in study II where other flow values were used for the kidney. Thus the pump was adjusted to deliver about 70 ml/min \times 100 g to the renal vascular bed (study II) while the flow to the hindquarters was about 10 ml/min \times 100 g (study III V). After having secured complete smooth muscle relaxation with bolus injections of papaverine

as mentioned above the perfusion was continued for some 15-20 minutes before NA infusion was started in order to completely wash out papaverine. NA dissolved in the perfusion medium was then infused in a stepwise fashion from subthreshold up to supramaximal concentrations. To induce definitely maximal pressor responses 0.5 - 1.0 mg of NA, 10 IU of vasopressin and 100 - 150 mg of BaCl_2 were finally added. The concentrations of vasoactive agents were always equal in the two preparations since the rats were of equal size, equal flows per unit tissue mass were used and the injections were made proximally to a mixing chamber from which the two inflow tubes to the animals emerged (see Fig. 2).

The perfusion pressure at maximal dilatation and the vasoconstrictor responses to NA, vasopressin and barium chloride were then calculated. Dose-response curves to NA were plotted for each paired experiment with μg of NA per ml perfusate on the (log) abscissa and perfusion pressure in mm Hg on the ordinate. Since flow rate was constant the resistance responses can be expressed by the perfusion pressure responses.

From these resistance curves the following key points were deduced and used when comparing the hemodynamic characteristics of the test animals to those of the controls:

1. Flow resistance at maximal dilatation: representing the structurally determined average luminal size of the resistance vessels.
2. The threshold concentration of NA, i.e. the NA concentration raising flow resistance 25 per cent above that at maximal dilatation as deduced from the curve. According to Poiseuille's law this would correspond to about 5 per cent shortening of the contractile elements. If the influence of the wall/lumen ratio is neglected which seems justified since even in the fairly thickwalled resistance vessels this ratio is low at maximal dilatation. The NA threshold concentration represents the NA sensitivity of the smooth muscle cells.
3. M_{50} , i.e. the NA concentration producing 50 per cent of the maximal NA pressor response. M_{50} is not identical with ED_{50} as used in conventional dose-response curves for a g. smooth muscle strips. The reason is that the increasing wall/lumen ratio of constricting vessels in an accelerated way potentiates the luminal reduction for a given degree of smooth muscle shortening. Further M_{50} is displaced to the left if one vessel is more thickwalled than the other even if their smooth muscle sensitivity is equal (cf. Fig. 1, right part).
4. The slope of the steep part of the resistance curve, i.e. the tangent of the angle which reflects the wall/lumen ratio as long as the degree

RESULTS AND COMMENTS

Structural changes of the total systemic vascular bed in SHR (paper 1)

In this study SHR in the phase of established hypertension were used to explore whether the adaptive vascular changes found in regional vascular beds in man with essential hypertension were present also in SHR and in that case if they were generalized throughout the systemic circulation. Perfusion of the entire systemic vascular bed (except the coronaries) of SHR and NCR showed that even during maximal dilatation systemic resistance was raised in SHR almost in proportion to the raised arterial pressure. As can be seen in Fig 3 which presents the mean pressure-flow curves of the maximally dilated systemic vascular beds flow resistance is throughout significantly increased in SHR compared to NCR. The difference between SHR and NCR is particularly evident at the higher range of pressures which is in line with subsequent findings that the SHR resistance vessels are less distensible than the NCR ones (Hallböök, Lundgren and Weiss 1974).

These results are not in agreement with those of Haessler and Finch (1972) which did not reveal any difference in resistance at maximal dilatation between SHR and NCR. However, there are two important differences between these two studies which may readily explain the apparently diverging results. First in the present study 7 month old rats with well established hypertension were used while Haessler and Finch used rats which being only 14 week old were in an earlier phase of hypertension where the structural vascular changes may not yet be fully developed. Second in the present study flow was changed between 3 and 40 ml/min $\times 100$ g while in the study by Haessler and Finch flow was not increased beyond 12 ml/min $\times 100$ g. As seen in the present results however the difference in flow resistance is small at very low flows and distending pressures but increases at higher flows and perfusion pressures which reflects a reduced vascular distensibility in SHR.

The present results indicate that the earlier mentioned structural adaptation of the resistance vessels in primary hypertension is on the whole generalized throughout the systemic vascular bed and almost in proportion to the raised arterial pressure. It follows that the raised flow resistance in SHR with established hypertension may to a great extent be accounted for by such a structural change, since it would reset the baseline for resistance vessel control to a higher level.

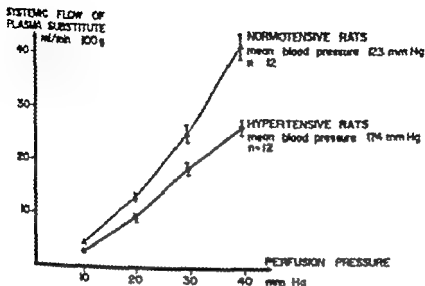


Fig 3 Mean pressure-flow curves from 24 perfusion experiments on the maximally dilated systemic vascular beds of 12 spontaneously hypertensive rats (SHR) and 12 normotensive control rats (NCR). Vertical bars indicate the standard error of the mean. The two curves are significantly separated ($p < 0.001$).

Structural changes of the SHR renal vascular bed (paper II)

Paired perfusions of the renal vascular beds of 7 month old SHR (established phase of hypertension) and matched NCR showed that this particular circuit exhibited a lower resistance at maximal dilatation in SHR compared with NCR. In other words the renal vascular bed differs in this respect from the entire systemic vascular bed (study I) and the hindquarter vascular bed (Folkow *et al.* 1970) in SHR as well as from the hand and forearm vascular beds in man with essential hypertension (Folkow, Grimby and Thulesius 1958, Conway 1963, Sivertsson 1970). However the SHR renal resistance vessels also display a reduced distensibility compared to those of NCR suggesting that the wider lumina are associated with thicker vascular walls as in other hypertensive circuits (cf H. Ilback, Lundgren and Weiss 1974).

Further Fig 4 illustrates that NA infusions during constant flow conditions revealed a steeper dose-response curve and an increased

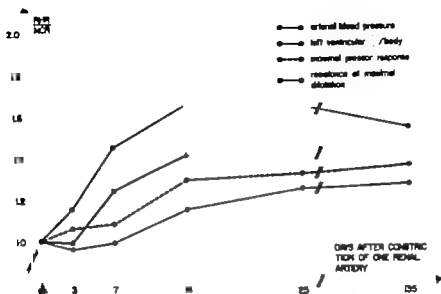


Fig 5 Time course and extent of adaptive cardiovascular changes in normotensive Wistar rats made hypertensive by renal artery constriction with days after artery constriction on the abscissa and on the ordinate the ratio between renal hypertensive rats (RHR) and normotensive control rats (NCR) concerning 1) arterial blood pressure (measured in the caudal artery during awake conditions), 2) left ventricular weight/body weight 3) maximal pressor response and 4) resistance at maximal dilatation (flow = 30 ml/min \times 100 g). Note that the adaptive hypertrophic changes of the left heart ventricle and the systemic resistance vessels are almost completed in RHR after 2-3 weeks. The reduced pressure ratio at 135 days is due to a slight increase of NCR pressure.

Rate and extent of development of adaptive cardiovascular changes in RHR (paper III)

In this investigation the extent and exact time course of cardiovascular structural adaptation to a rapid and fairly extensive rise in mean arterial pressure were studied after induction of renovascular hypertension in normotensive rats. At different time intervals after renal artery constriction the hemodynamic characteristics of the RHR and NCR hind-

quarter vascular beds were explored from maximal dilatation up to maximal constriction. Also the extent of left ventricular hypertrophy in RHR was examined. The results revealed the presence of left ventricular hypertrophy in RHR already after one week, closely followed by adaptive structural changes of the RHR resistance vessels. Fig. 5 illustrates the time course of these changes with days after renal artery constriction on the abscissa and on the ordinate is given the ratio RHR/NCR concerning arterial pressure, left ventricular weight/body weight, maximal pressor response and flow resistance at maximal dilatation. The cardiovascular structural adaptation appears to be largely completed 2-3 weeks after operation, since no further significant increase in blood pressure, left ventricular hypertrophy or media hypertrophy appears to occur. Thus, compared to NCR the rats with established renal hypertension (25 days after operation) display in essence the same changes concerning their resistance curves as illustrated for SHR in the left part of Fig. 1 (a, 1) an increased resistance at maximal dilatation, 2) a steeper slope of the resistance curve and 3) an increased maximal pressor response, but no change in NA sensitivity (Fig. 6). There is always a bigger resistance increase in RHR than in NCR for a given NA concentration, which would be expected to produce largely the same extent of smooth muscle activation (as can be seen in Fig. 6). These results from RHR 25 days after operation are in agreement with findings on DOCA or renal hypertensive animals (Overbeck et al. 1971, Bellin and Zlokovic 1972, Haessler and Finch 1972) with the exception that the latter investigators also reported an increased NA sensitivity of the resistance vessels in renal and DOCA hypertensive rats.

It is seen in Fig. 5 that the structural cardiovascular adaptation starts before the pressure rise is completed. Thus, this structural adaptation is evidently so rapid that e.g. its hemodynamic consequences in terms of both baroreceptor resetting and resistance rises for given smooth muscle activity levels are likely to become intertwined in time with the functional pressor influences which evidently act as trigger mechanisms for the structural changes. This makes it probable that the very creation of the raised pressure is a matter of both functional and structural processes in close cooperation. In man such structural changes are no doubt slower than in rats, mainly because of the lower metabolic rate, but the development of hypertrophic changes is likely to be quite rapid also in man. In fact, an increased flow resistance even during maximal dilatation is observed in the hand of young men (about 20 years old) with borderline hypertension as compared to sex and age matched normotensive controls (Sjörinsson, Sannerstedt and Lundgren, preliminary results).

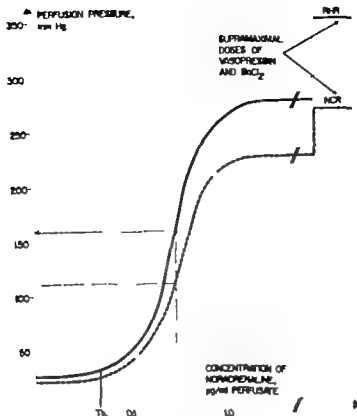


Fig. 6 Mean resistance curves based on 12 constant flow perfusions paired hindquarter vascula beds from renal hypertensive rats (RHR) 25 days after renal artery constriction (mean arterial pressure 180 ± 6 mm Hg) and normotensive control rats (NCR; mean arterial pressure 111 ± 3 mm Hg) showing the perfusion pressure (resistance) responses to increasing concentrations of noradrenaline (NA) from subthreshold up to supramaximal amounts. Maximal pressor responses were ensured by huge doses of vasopressin and barium chloride. NA concentration ($\mu\text{g/ml}$ perfusate) is plotted along the abscissa on a log scale and perfusion pressure responses (mm Hg) along the ordinate. Th denotes threshold, i.e. the NA concentration producing 25 per cent increase of resistance above the state of maximal dilatation.

Note the increased resistance already at maximal dilatation in RHR, the enhanced steepness of the resistance curve and the increased maximal

pressor response while there is no difference in NA threshold dose between RHR and NCR. The thin dotted lines illustrate how resistance to flow becomes higher in RHR than in NCR for the same NA concentration i.e. the same degree of smooth muscle activation.

135 days after operation there was a slight further increase in resistance at maximal dilatation and in steepness of the RHR dose response curve compared to RHR 25 days after operation. However no further increase in maximal pressor response was noted. These delayed changes might suggest a slight further increase in wall thickness that may not be due to an increase in smooth muscle tissue but in e.g. water or/and collagen content. In fact an increased amount of collagen in the aorta has been demonstrated in long-term compared to short term renal hypertension (Wolinsky 1972). Concerning water logging (Tobian and Blinco 1952, Tobian, Olson and Chesley 1969, Tobian 1972) the present results reveal a significantly increased water content of the RHR aortic walls 135 days after operation compared to matched NCR and/or RHR 25 days after operation but no difference was found between RHR 25 days after operation and their matched NCR. From these results which however are obtained from the aorta it seems possible that both collagen invasion and some water logging may contribute somewhat to the wall thickening also in the resistance vessels in later phases of hypertension though the hypertrophic adaptation seems to be by far the most rapid extensive and therefore hemodynamically important structural change in these vessels.

The present results illustrate in principle that the cardiovascular structural adaptation to increased pressure occurs not only in animals genetically predisposed to hypertension like SHR but also in genetically normotensive animals in which hypertension is induced by interference with e.g. the renal blood supply (RHR). However even if the same kind of cardiovascular change is present in both types of hypertension, this does not exclude the possibility of a quantitative difference i.e. the cardiovascular structures in primary hypertension may respond with somewhat more pronounced changes in design when exposed to a given pressure load. In case such a difference really exists it is not necessarily inherent in the cardiovascular tissues per se; it may as well be a consequence of some genetically linked difference in e.g. hormonal mechanisms that facilitate the development of hypertrophic changes.

A comparison has been made between SHR (8 months old) and RHR (135 days after operation, 6 months old) where the hypertensive state is of approximately the same duration. The arterial pressure levels were related to left ventricular weight and to maximal contractile strength.

of the resistance vessels (Folkow et al 1973). In order to investigate to what extent the hypertrophic changes were in balance with the load to which heart and resistance vessels were exposed. When comparing SHR and RHR in this way the hypertrophic changes appeared to be somewhat more pronounced in SHR than in RHR. If resting arterial pressure can be considered as a reflection of the average pressure load. This is however one of the problems in hypertensive research in general because it is in reality not known exactly what the average pressure is during days and weeks since this would call for long-term continuous pressure measurements in normal daily life. No doubt the present results may support the view that SHR display a more pronounced cardiovascular hypertrophy than RHR for a given load but to prove this is hardly possible at present. It might for example also be so that the true average pressure in SHR is in reality higher than the measured resting pressure level would indicate while this latter level better corresponds to the average pressure in RHR. Thus SHR might display pronounced and frequent pressure enhancements as a result of transient neurohormonal discharges in daily life superimposing an extra pressure load which may be far less pronounced in RHR. No doubt the SHR pressor responses to acute alerting stimuli are more pronounced than in RHR (Hallböök and Folkow 1974). If this latter alternative is of relevance it would however in itself imply an important and interesting difference between primary and secondary hypertension.

Studies where renal hypertension has been provoked in both young SHR and young NCR are in progress as an attempt to shed further light on the problem whether the cardiovascular changes are more pronounced in primary hypertension than in renal hypertension but it is still too early to draw any conclusions. In any case it appears as this structural autoregulation is so pronounced in SHR as to largely explain the raised resistance during rest, while this is hardly the case in RHR where functional excitatory influences evidently must contribute more strongly to maintain the raised resistance and pressure levels. However, the question if the hypertrophic cardiovascular changes are more pronounced in SHR than in RHR is far from settled and more studies are needed to solve this problem.

Regression of hypertensive cardiovascular changes upon reversal of renal hypertension in rats (paper IV)

To study how rapidly and to what extent regression of the hypertrophic cardiovascular changes in renal hypertension can occur the renal artery clip was removed 3-4 weeks after clipping. Arterial pressure had

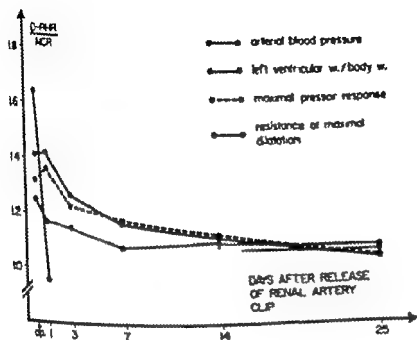


Fig 7 Time course and extent of regression of the hypertensive changes in cardiovascular design after reversal of renal hypertension. Days after declipping are plotted along the abscissa and along the ordinate the ratios between renal hypertensive rats (RHR) before and after (D-RHR) declipping and their normotensive control rats (NCR) concerning 1) arterial pressure 2) left ventricular weight/body weight 3) maximal pressor response and 4) flow resistance at maximal dilatation (Flow = 30 ml/min \times 100 g). Thus the cardiovascular system still displays structural hypertensive changes one day after declipping but rapid regression then occurs with normalization after about 3 weeks.

then stabilized at about 180 mm Hg and according to the previous study the structural adaptation was fully developed. Paired hindquarter perfusions were performed on one declipped RHR (D-RHR) and one normotensive control rat (NCR) 1 3 7 14 and 25 days after releasing the clip thus exploring the differences between them concerning resistance vessel design. On the same occasions the regression of cardiac hypertrophy was followed by measuring left ventricular weights. Blood pressure was normalized already after one day but no signi-

ficant regression of the hypertensive structural changes could be traced neither 1 nor 3 days after unclamping. Then however both the cardiac and vascular hypertrophic changes rapidly diminished and were largely eliminated after 3 weeks as illustrated in Fig 7. In this Fig days after release of the renal artery clip are given along the abscissa while the ordinate gives the ratios between D-RHR and NCR concerning arterial pressure, left ventricular weight/body weight, maximal pressor response and resistance at maximal dilatation. Resistance at maximal dilatation appears to be the parameter most rapidly normalized, i.e. almost within 1 week, while the increased steepness of the D-RHR resistance curve, the maximal pressor response and the left ventricular weight were largely normalized in 3 weeks, indicating a regression of media and cardiac hypertrophy.

The early decrease of resistance at maximal dilatation implies only some 4-5 per cent increase in average internal radius, negligibly affecting wall tension according to Laplace's law. This effect on wall tension is completely overcome by the far bigger fall in transmural pressure. However, this nevertheless illustrates that the internal radius can change relatively independently of wall thickness. Evidently wall thickness adjusts more or less precisely to wall tension, while internal radius might also respond to other elements, such as the local chemical environment, as determined by the balance between tissue metabolism and its blood supply.

The present results are in agreement with studies on cat and SHR where pressure reductions after arterial obstructions or hypotensive drug treatment induced rapid changes of resistance vessel design (Folkow and Silverstén 1968, Folkow et al 1971 b, Weis 1974, Weis and Böck 1974). Further, immunosympathectomy in newborn SHR or aortic

in young SHR largely prevents the development of the mentioned hypertensive vascular changes (Folkow et al 1971 a, Folkow et al 1972). Together these studies suggest that the hypertrophic cardiovascular changes mainly represent a regional structural adaptation to the average pressure load.

Likewise, morphological and chemical studies of the rat thoracic aorta (Walinsky 1971) reveal a considerable decrease of the smooth muscle component after reversal of renal hypertension. However, in these studies the duration of hypertension was longer than in the present study (10 weeks compared with 3-4 weeks) and there was no complete regression of media thickness, at least not in male rats. The reason was mainly that there was also a considerable increase of elastin and collagen in the aortic wall and these two substances showed very poor regression upon pressure reduction. Such elements may increase also in the resistance vessels proper with time, even though their rela-

the proportions are here normally much smaller. However, once a substantial collagen invasion has occurred also in the resistance vessels its regression is likely to be sluggish as in the aorta. This may explain why Tobian, Olson and Chesley (1969) did not observe any decrease in mesenteric arteriolar wall thickness after reversal of long standing renal hypertension.

The results of the present study together with those of e.g. Tobian, Olson and Chesley (1969), Wollinsky (1971, 1972) and Weiss (1974) illustrate the advantage of early antihypertensive therapy before accumulation of elastin and collagen occurs since the earlier established hypertrophic changes seem to disappear completely and rapidly. Once the less reversible changes, e.g. accumulation of collagen, are established both resetting of the baroreceptor mechanisms to normotensive levels (cf. Aars 1969) and readjustment of resistance vessel dynamics would be far more difficult to achieve by antihypertensive treatment.

The present results confirm the findings by e.g. Byrom and Dodson (1949), Floyer (1955), Ledingham and Cohen (1962), Liard and Peters (1970) and Funder et al. (1970). Thus blood pressure in relatively early renal hypertension in rats is rapidly normalized after release of the renal artery constriction. This occurs despite the presence of considerable hypertrophic cardiovascular changes. According to Ledingham (1971) this rapid pressure normalization is the result of a sharp reduction in cardiac output while resistance further increases. If anything at least in short term measurements (6 hours). The hemodynamic situation is however extremely complicated; for example the sudden overperfusion of the earlier obstructed low pressure kidney may initiate complex neurohormonal shifts where among other possibilities depressor substances from the kidney in the form of prostaglandins (e.g. Tobian 1972) etc. may be of importance. Further the neurogenic tonic influences might become subnormal as a result of the suddenly changed renin-angiotensin equilibrium. However very little is so far known about these events and their background and more penetrating studies are obviously needed.

If in well established renal hypertension the clipped kidney is instead extirpated the hypertensive state tends to prevail (Wilson and Byrom 1941, Pickering 1945, Floyer 1955, Funder et al. 1970) indicating extrarenal pressor mechanisms. However extirpation of the ischemic kidney after short term hypertension (0-8 days) brings about an initial decrease in blood pressure (Pickering 1945). It is possible that the presently studied structural autoregulation of the resistance vessels may at least partly account for the extrarenal pressor mechanisms. On the other hand preliminary results from this laboratory

reveal an immediate normalization of blood pressure when the ischemic kidney is extirpated 4 weeks after renal artery constriction

Obviously the results in this field are still confusing and in part contradictory calling for more penetrating studies of e.g. neurogenic control the balance between cardiac output resistance and capacitance vessel control etc. The sudden release of the renal artery constriction or extirpation of the clipped kidney with a consequent loss of powerful humoral drive mechanisms are likely to induce gross though transient changes in neurohormonal cardiovascular control. If so the consequent depressor effects would counteract the impact of already established structural changes and allow for their gradual elimination. Thus whatever the mechanism behind the normalization of blood pressure in early renal hypertension the reduced pressure level will lead to a rapid and complete regression of the structural cardiovascular changes and hence to a stabilization of the normotensive state

Propranolol treatment in RHR (paper V)

It was explored whether preventive treatment with β -receptor antagonists would interfere with the development of renal hypertension and the hypertrophic vascular changes first because neurogenic elements and cardiac output increases have been much discussed also in early renal hypertension (cf. Page and McCubbin 1968 Guyton and Coleman 1969 Ledingham 1971) and second because such treatment effectively prevents the progress of SHR hypertension (Folkow Lundgren and Weiss 1972 Weiss Lundgren and Folkow 1974)

The results of the present study indicate that in contrast to the situation in young SHR renal hypertension develops just as ly despite preventive treatment with propranolol. In concentrations would efficiently block the adrenergically mediated renin secretion (Sykes et al 1970 Bühler et al 1972 Michelakis and McAllister 1972) as well as the adrenergic control of the heart and thereby decrease the cardiac output (Frolich et al 1968 Ulrych et al 1968). However the exact mechanisms of β -adrenergic receptor antagonists are not yet known and it is possible that they exert one of their main effects via the central nervous system (Sannerstedt 1974). Paired hindquarter perfusions revealed the same degree of hypertensive structural changes as in untreated RHR controls. Further prolonged propranolol treatment in rats with already established renal hypertension did not affect arterial pressure. Therefore the present results do not suggest any major importance of the adrenergically mediated increases of cardiac output or/and enin release neither for the development nor for the maintenance of renal hypertension. The difference between these

results in RHR and those in early SHR hypertension also supports the view that the initiating mechanisms must be different in these two types of hypertension.

GENERAL DISCUSSION

Adaptive structural changes of the resistance vessels mainly in the form of an increased media thickness associated with a luminal reduction have been demonstrated in regional vascular beds in man with essential hypertension as well as in SHR (cf Folkow et al 1973). Like left ventricular hypertrophy and similar changes in larger arteries this phenomenon may be considered as a basically normal response of mesodermal tissues to increases in functional load thus occurring in any type of sustained increase of average pressure the reverse process taking place upon pressure reductions. These secondary structural adjustments of the cardiovascular system occur so rapidly (study III) that their hemodynamic consequences in terms of e.g. the resistance rise for a given smooth muscle activity level or the resetting of baroreceptors (cf Aars 1969) are likely to become intertwined in time with the functional pressor influences which serve to trigger the structural adaptation. Hence these changes are likely to contribute not only to the maintenance but also to the very creation of a chronic hypertensive state. In rats they are almost completed in 3 weeks though in man the slower metabolism is likely to imply a considerably longer period. However even if the development of hypertrophic adaptation is slower in man it is still a relatively rapid process considering the gradual onset of essential hypertension and the far longer life span in man.

The structural vascular changes are generalized occurring in the entire systemic vascular bed as shown in study I. However when comparing different systemic circuits they seem to differ somewhat in of luminal dimensions at maximal dilatation (study II). Thus the renal circuit displays a decreased flow resistance during maximal dilatation at least at relatively low transmural pressures while the vascular bed of e.g. the skeletal muscles and the average of the systemic vascular bed then display an increased resistance. This difference might be a reflection of corresponding differences in basal vascular tone precapillary smooth muscle tone being normally very low in the kidneys but quite high in e.g. skeletal muscle implying that the structural vascular adaptation in such circuits is initiated from quite different levels of functionally determined wall/lumen ratios. In this respect the situation in the renal resistance vessels is related to that in most conduit arteries where smooth muscle tone is usually low and where the adaptive wall thickening is also usually associated with an increased inner radius. However common to all vessels exposed to an increased average pressure load seems to be the increased wall thickness leading to a raised wall/lumen ratio (see also Furuyama

1962 Suwa and Takahashi 1971)

Changes of this general nature are indeed biologically most appropriate, e.g. for inducing structural adaptations also in the normal vascular bed. Thus vessels exposed to enhanced transmural pressures as a result of hydrostatic influences, e.g. the veins of the lower limbs, tend to increase their wall thickness (Jones and Dale 1958, Svejcar et al 1962). The same is true in pulmonary hypertension (Ferguson and Varco 1955). With respect to the resistance vessels these locally induced structural adjustments may be called structural autoregulation as a more slowly developed correlate to the acute functional autoregulation displayed by most resistance vessels as a local response to sudden pressure changes. Like this process it implies however a type of positive feedback mechanism and therefore a potential risk factor particularly when it affects the systemic vascular bed as a whole. The reason is that it tends to set the pressure-resistance equilibrium to a higher level and thereby increasing the chances of degenerative and lesional changes and thus true disease.

An important question is if animals with primary hypertension (SHR) are perhaps genetically more prone to respond with cardiovascular hypertrophy to a given increase in pressure load than those with secondary hypertension (RHR). Even a modest quantitative difference in this respect is important since it may in the long run have considerable hemodynamic consequences because of the tendency of the functional and structural factors to reinforce each other with respect to resistance vessel control. From the present study it appears as if the extent of structural change of heart and resistance vessels may be somewhat more pronounced in SHR than in RHR for a given level of resting arterial pressure. As a possible parallel it should be recalled that patients with essential hypertension have a prevalence of mesomorphic body build (Robinson and Bruce 1940) which with respect to heart and blood vessels might imply a greater tendency to respond structurally to a pressure load as may be the case in SHR. However as discussed earlier this problem is very difficult to settle and the evidence so far should rather be considered as indicative.

Another important question is: What initiates these structural changes of heart and resistance vessels? Primary and renal hypertension will here be discussed separately since fundamental differences evidently exist between these two types of hypertension concerning the nature of the functional initiating mechanisms.

Primary hypertension

The strong hereditary element in this important disorder of regulation is well known and seems to be made up of several genetically linked factors both in man and in rats (Pickering 1968 Okamoto 1972). As previously mentioned the observed structural cardiovascular changes are secondary in nature though rapidly established and perhaps particularly easy to induce in subjects predisposed for hypertension. The establishment calls for trigger factors in terms of functional increases of the average pressure load continuous or intermittent in nature. It is however unlikely that such a pressure load would be of renal origin in SHR (see Okamoto 1972) and in study II it is shown that there is nothing to indicate any structurally based Goldblatt mechanism in early SHR hypertension.

It has been proposed that primary hypertension in man might be due to a complex interaction between centrally elicited increases of arterial pressure and cardiac output with a gradual structural adaptation of heart and precapillary vessels to such functionally elicited pressure increases (Folkow 1960). The characteristic hypothalamic defence reaction (e.g. Eliasson *et al* 1951) conveying most such responses elicited by psychogenic stimuli is present throughout the animal species including man (e.g. Folkow 1960 Brod *et al* 1962 Brod 1963 Charvat, Dell and Folkow 1964 Folkow and Rubinstein 1966 Henry Meehan and Stephens 1967 Folkow and Neil 1971). In fact weak but often repeated stimulations of the hypothalamic defence area in rats result in a moderate but fairly sustained hypertension (Folkow and Rubinstein 1966) as is the case also when mice and monkeys are exposed to environmental stress (Henry Meehan and Stephens 1967 Herd *et al* 1967).

Also in man the characteristic defence reaction induced by strain of daily life can lead to often considerable although transient increases in cardiac output and blood pressure (Brod 1960 Hines, Engel and Bickford 1962 Pickering 1968 Bevan Honour and Stott 1969). In fact early labile stages of essential hypertension usually display a cardiovascular pattern closely mimicking a mild defence reaction (e.g. Brod 1963 Sannerstedt 1966 Julius Pascual and London 1971). Thus it seems as the central discharge pattern to ordinary alerting situations is accentuated in subjects developing essential hypertension (e.g. Hines and Brown 1933 Kalls *et al* 1957 Folkow 1960 Charvat, Dell and Folkow 1964 Nestel 1969 Lorimer *et al* 1971).

A long series of studies by Okamoto's group (cf. Okamoto 1969 1972) provides evidence of an enhanced neurohormonal discharge to the cardiovascular system in SHR. Further also young prehypertensive

SHR display an inherent evidently genetically linked hyperreactivity of the autonomic centers which during alertness and mental stress reflects increased changes in cardiovascular function (Hallböök and Folkow 1974). Even if largely intermittent in nature, such influences may well serve to initiate structural cardiovascular adaptation as discussed above. Such an inherent hyperreactivity of central autonomic structures will also be greatly influenced by the environmental situation being further potentiated by stress thus implying a complex interaction between genetic and environmental factors.

The importance of neurogenic mechanisms for initiation of hypertension in SHR is also evident by the effects of immunosympathectomy in anorectic SHR (Clark 1971, Folkow et al 1972) and of prolonged treatment with β -adrenergic receptor antagonists and other sympatholytic drugs in young SHR (Weiss 1974, Weiss, Lundgren and Folkow 1974). All these procedures interfere more or less drastically with the development of spontaneous hypertension and the associated vascular changes.

In other words, both in SHR and in man with essential hypertension there may be a combination of increased central neuro-hormonal reactivity and secondary, although rapidly developed, structural cardiovascular changes, the latter being a response to the enhanced average pressure load. Both of these factors are *per se* perfectly normal mechanisms which, however, may for genetic reasons be somewhat enhanced in extent in SHR and perhaps also in man with essential hypertension. However, the situation is in all probability far more complex than so, because also other elements are likely to be involved and the balance between them may vary from individual to individual at least in man where the genetic background is far more heterogeneous than in the inbred SHR.

Renal hypertension

The background of the initial increase in blood pressure in renal hypertension is still far from clear despite great experimental efforts. No doubt renal humoral mechanisms are of primary importance but they may to a great extent exert their cardiovascular effects via neurogenic links (Dickinson and Yu 1967, Ueda et al 1969, Joy 1971, Ferraris, Gildenberg and McCubbin 1972) as outlined earlier. In initial phases there is often a transient increase of cardiac output noted by several investigators (cf Ledingham 1971, Bianchi et al 1972). However, this proposed increase in cardiac output is then not only a result of the adrenergic myocardial innervation since prolonged treatment...

the adrenergic β -receptor antagonist propranolol did in no way interfere with the development of renal hypertension or the hypertensive vascular changes (study V). The initiating renal mechanisms are evidently capable of inducing hypertension also via other links of the autonomic innervation in cooperation with e.g. direct influences on the vessels. Interferences with the sodium balance via aldosterone etc. In fact the importance of the sympathetic nervous system for the development of renal hypertension is still not clear. Thus Finch and Leach (1970) concluded that renal as well as DOCA hypertension can develop even though most of the sympathetic nervous system has been destroyed while Grewal and Kaul (1971) and Ayitey-Smith and Varma (1970) concluded that at least some aspects of the sympathetic control of the cardiovascular system are essential for the development of renal hypertension.

Part of the renin secretion (cf. Davis 1973) is mediated via adrenergic β -receptors (Vandongen, Peart and Boyd 1973) but probably not that part which is induced by regional hypotension and relative ischemia of the kidney (e.g. Blaine and Davis 1971). The fact that propranolol treatment which seems to inhibit the neurogenic control of renin secretion in no way prevents the development of renal hypertension (study V) indicates that the other local mechanism for renin secretion is the by far dominating one for eliciting renal hypertension. Further immunization against angiotensin does not prevent the development of renal hypertension (e.g. Elde and Aars 1970, Johnston, Hutchinson and Mendelsohn 1970, MacDonald et al 1970, Elde 1972) indicating either that the kidneys possess other means for raising arterial pressure when their perfusion is threatened or that the immunization was not capable of suppressing all angiotensin, e.g. the facilitating effect on sympathetic discharge via bulbar efferent fibers.

The fact that pressure promptly falls to normal or even subnormal levels after release of the renal artery constriction (and even sometimes after extirpation of the ischemic kidney), despite the presence of cardiovascular hypertrophic changes (study IV) indicates that cardiac output or/and vascular tone must then become subnormal. The sudden elimination of renal humoral excitatory influences perhaps associated with an excess release of depressor humoral agents like prostaglandins etc. from medullary interstitial cells (e.g. Tobia 1972) along with the overperfusion of the earlier ischemic kidney may here be of great importance. Such a subnormal vascular tone and neurogenic drive on the system will if prolonged enough not only normalize blood pressure even if extensive structural cardiovascular changes are present but also induce regression of these changes. However such a proposed sequence of

mechanisms is difficult to follow in detail experimentally. For such reasons very little is so far known about the true events which whatever their nature are of great importance for understanding renal hypertension.

Regression of cardiovascular changes

The present study (IV) has shown that a rapid regression of cardiac as well as media hypertrophy occurs after normalization of blood pressure at least in early renal hypertension. Thus all cardiovascular changes are eliminated after 3-4 weeks of normotension. The same general time course is seen in cats or in SHR when only regional hypotension is induced (Folkow and Silverstam 1968, Weiss and Hallböök 1974). Further intensive treatment of SHR with hypotensive drugs leads to considerable regression of these changes already after 5 weeks although this regression appears to become slower and less complete the more advanced the hypertensive state (Folkow et al 1971 b, Weiss 1974). The slower and less complete regression of the vascular changes in long-term hypertension may reflect that the rapidly established smooth muscle hypertrophy in the resistance vessels is gradually superimposed by or/and transformed into structural changes of a more degenerative nature such as collagen invasion which are less liable to exhibit regression than smooth muscle hypertrophy. A parallel may here be drawn to the aorta where it has been observed that increased amounts of collagen and elastin are formed in long-term hypertension and that these wall elements show far less regression than the smooth muscles upon reversal of renal hypertension (Wollinsky 1971, 1972).

Together these results illustrate the advantage of an early induction of antihypertensive therapy when the structural changes are still mainly in the phase of muscle hypertrophy. Thus if blood pressure is normalized in this early phase the hypertrophic changes of the resistance vessels and also of the left ventricle and probably large arteries are likely to disappear completely and rapidly. The tendency of a vicious circle inherent in the potentiation between adaptive hypertrophic changes and functional excitatory influences in terms of resistance vessel responses will then also be interrupted. Once more persistent changes have been added such as collagen invasion in heart arteries and resistance vessels these changes also increasing wall thickness would tend to remain decreasing the chances for inducing a true reversal of the hypertension state. Thus the resistance vessels would then still tend to exhibit exaggerated luminal reductions upon smooth muscle contractions and the stiffer walls at the site of the arterial baroreceptors would interfere with their resetting to the normal pressure range (cf Aars 1969).

SUMMARY OF THE PRESENT RESULTS

The results obtained in these comparative hemodynamic studies on spontaneously hypertensive rats (SHR) renal hypertensive rats (RHR) and normotensive control rats (NCR) can be summarized as follows:

- I Adaptive structural changes of the resistance vessels are largely generalized throughout the systemic vascular bed in SHR. They seem to be largely in proportion to the raised arterial pressure but are evidently mainly confined to the precapillary vascular sections to judge from still more recent findings.
- II No structurally based Goldblatt mechanism seems to be involved in the establishment of SHR primary hypertension. Thus the SHR renal vascular bed displays a reduced resistance to flow during maximal dilatation. In contrast to most other systemic circuits in SHR. However like the situation in these other systemic circuits the SHR renal resistance vessels are characterized by an increased wall/lumen ratio to judge from their hemodynamic characteristics.
- III The cardiovascular structural adaptation to increases in pressure load is qualitatively equal in rats with genetically linked hypertension (SHR) and in genetically normotensive rats (RHR) but might be quantitatively somewhat more pronounced in SHR than in RHR. The process is a very rapid one with respect to the development of media and left ventricular hypertrophy being completed about 3 weeks after initiation of renal hypertension in rats. Vascular wall lagging appears to occur only in late phases of hypertension and seems even then to be of far less hemodynamic significance than the structurally based increase of wall thickness.
- IV After reversal of early renal hypertension the hypertrophic cardiovascular changes are hardly significantly changed during the very first few days. Then however a rapid gradual regression occurs so that a normalization of the design of heart and resistance vessels appears to occur after 3-4 weeks of normotension.
- V Propranolol treatment effectively interfering with the development of SHR hypertension has no similar preventive effects either on the development of renal hypertension or the hypertensive vascular changes. Further no hypotensive effect of prolonged propranolol treatment was found on already established renal hypertension.

The mentioned findings in SHR, RHR and NCR are discussed with

particular reference to the interactions between functional and structural changes affecting resistance vessel control in primary and secondary hypertension

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**ASPECTS OF THE RELATION BETWEEN FUNCTIONAL AND
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Experimental studies in spontaneously hypertensive rats

**BY
LILIAN WEISS**

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University of Göteborg, Sweden**

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- I The hemodynamic consequences of regional hypotension in spontaneously hypertensive and normotensive rats
B Folkow M Gurévich M Hallböök Y Lundgren and L Weiss Acta physiol scand 1971 83 532-541
- II Time course and extent of structural vascular adaptation to regional hypotension in adult spontaneously hypertensive rats (SHR)
L Weiss and M Hallböök Acta physiol scand In press
- III The effects of immunosympathectomy on blood pressure and vascular reactivity in normal and spontaneously hypertensive rats
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- V Long-term treatment with antihypertensive drugs in spontaneously hypertensive rats (SHR) Effects on blood pressure survival rate and cardiovascular design
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The papers are referred to in the text by their Roman numerals

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INTRODUCTION

Despite much effort the exact background of essential (primary) hypertension is not yet understood. The first tentative description of this important disorder of regulation was presented more than 200 years ago (cf Backer 1953). However, it was not until the presence of an increased intravascular pressure was demonstrated in the late 19th century (e.g. Mahomed 1881) that more extensive investigations were made possible. The probable importance of genetic influences was suggested by Morgagni already in 1769 (cf Pickering 1968) but not until fairly recently have epidemiological studies clearly revealed the involvement of a polygenic inheritance (cf Pickering 1968, Miall 1971).

The present investigation was performed on a strain of rats with primary hypertension, i.e. the spontaneously hypertensive rat (SHR; Okamoto and Aoki 1963, Okamoto 1969) which is so far considered to be the most appropriate animal model of essential hypertension in man. Similar to human hypertension a polygenic inheritance has been shown in these hypertensive rats. Furthermore they display the same type of cardiovascular sequelae as is seen in man (Okamoto 1972). Interest was focused primarily on the structural cardiovascular adaptation of the systemic resistance vessels. Its development and regression and its hemodynamic importance since this part of the cardiovascular system constitutes the main point of disturbance once hypertension has reached its established phase. For these purposes a hemodynamic approach was used for quantitative evaluation of the vascular changes.

Mean arterial pressure is determined by cardiac output and peripheral resistance and an increased pressure may be induced by increases of one or both of these factors. Furthermore a increased resistance might be due to an increased length of the vessels, to an increased blood viscosity and/or to a reduced cross-sectional area of the vascular bed. Below these different possibilities will be briefly outlined.

PREVIOUS STUDIES

Cardiac output

It has been suggested that an increased cardiac output might play an important role particularly for the initiation of essential hypertension. Thus Wezler and Böger (1939), Werkö and Lagerlöf (1949) and Varnauskas (1955) observed an increased cardiac output in combination with

a largely normal peripheral resistance in hypertensive subjects. These findings have been further confirmed and suggested to be present in early phases of the disease (e.g. Finkelstein, Worcel and Agrest 1965, Sannerstedt 1966, Lund-Johansen 1967, Julius and Conway 1968, Frohlich et al. 1970, Julius and Schack 1971). A cardiovascular pattern associated with an increased cardiac output may indicate the presence of an increased neurogenic influence similar to that of a mild defence reaction (Eliasson et al. 1951, Brod 1963, Charvat, Dell and Falkow 1964, Julius, Pascual and London 1971).

On the other hand, there is no evidence of an increased cardiac output in the established phase of hypertension (Goldring and Chasis 1944, Fraley 1960, Pickering 1968, Frohlich et al. 1970). Therefore the main discussion in the literature has concerned the problem of the increased resistance at this stage of the disease.

Resistance

So far no reliable studies have suggested that an increase in length of the systemic resistance vessels or a raised blood viscosity should play an important role for the well-known increase in resistance in established essential hypertension (Pickering 1968). However, an increased viscosity *in vitro* has been reported in patients with essential hypertension (Tibblin et al. 1966), but it should be pointed out that such measurements often poorly reflect the viscosity of blood *in vivo* (Djafarzadeh et al. 1970).

According to the law of Poiseuille, the resistance is inversely proportional to the fourth power of the internal radius of the vessel. Therefore changes in the internal radius have a far greater influence on resistance to flow than changes in either viscosity or vascular length. Most investigations have been concerned with the functional restrictions of the internal radius of the resistance vessels as induced by an increased vascular smooth muscle activity, while the hemodynamic influence of structural vascular changes have been almost totally neglected.

Decreased internal radius due to increased smooth muscle activity

Several possible mechanisms that may enhance vascular tone in hypertension are discussed in the literature: 1) increased vasoconstrictor fibre activity, 2) increased amounts of humoral vasoconstrictor agents, 3) increased smooth muscle sensitivity to vasoconstrictor influences, 4) increased myogenic tone and 5) changes in ion composition.

1) Increased vasoconstrictor fibre activity to the systemic resistance vessels of hypertensive patients during rest has not been clearly demon-

strated (Pickering 1936 Prinzmetal and Wilson 1936 Fels 1960 Pickering 1968 Wallin Dillius and Hogborth 1973). However more or less transient increases of sympathetic discharge to both heart and blood vessels during daily life might result in more powerful and/or frequent pressure rises in hypertensive than in normotensive subjects (Hirman Engel and Blackford 1962 Pickering 1968 Bevan Honour and Stott 1969). Such differences may at least sometimes reflect accentuated central discharge patterns to ordinary alerting situations (Hines and Brown 1933 Folkow 1960 Charvat Dell and Folkow 1964 Hallbeck and Folkow 1974). Recent findings of increased plasma catecholamines in patients with essential hypertension (Engelman Portnoy and Sjoerdsma 1970 DeQuattro and Chan 1972) may further support this hypothesis. Furthermore it has been shown that sustained hypertension can be induced by frequently repeated direct hypothalamic stimulations (Folkow and Rubinstein 1966) or when such activations are induced by stressful environment (e.g. Henry Meehan and Stephens 1967 Herd et al 1969 Gutman and Benson 1971).

2. Humoral vasoconstrictor agents may directly or indirectly induce hypertension and have been found in some special forms of hypertensive disease such as in pheochromocytoma and renal hypertension possibly also in Cushing's syndrome and Conn's disease. However such agents have not been convincingly demonstrated in uncomplicated primary hypertension (Pickering 1968 Okamoto 1972).

3. Increased sensitivity (reactivity) to vasoconstrictor influences also denoted as supersensitivity or hyperreactivity is a concept which is often poorly defined in the literature (e.g. Silverston 1970) and is sometimes indiscriminately used for a whole variety of possible differences in vascular design and effector sensitivity. It is necessary to distinguish between increased sensitivity or reactivity in the pharmacological sense i.e. true smooth muscle supersensitivity and the hemodynamic aspect of vascular reactivity where also the influence of the geometrical arrangement of the contractile elements in relation to the lumen must be considered (cf. Johansson 1974). Furthermore increased reactivity e.g. in the sense of an increased pressure response to a given stimulus which has been studied since the beginning of the century includes besides the possible increase in smooth muscle sensitivity and/or increased wall/lumen ratio also the complex pressure regulation of the entire cardiovascular system. To exemplify a reduced responsiveness of the homeostatic baroreceptor reflexes might result in a type of hyperreactivity to pressor influences etc. In general therefore the pressure responses to cold exposure (e.g. Hines and Brown 1933; cf. Pickering 1968 Silverston 1970) and the pressure and/or resistance

responses to standard doses of pressor substances (e.g. Clough 1920 Goldenberg et al 1948 Doyle and Black 1955 Freis 1960; cf Pickering 1968 Sivertsson 1970) have been extensively studied without providing conclusive information about the exact background of the enhanced responses frequently observed in essential hypertension (cf Sivertsson 1970). Nevertheless it has quite often been taken almost for granted that exaggerated vascular pressor responses should primarily reflect an increased smooth muscle sensitivity (reactivity) thus neglecting possible influences of a changed vascular structural design.

In order to explore the true smooth muscle sensitivity it is advantageous to study strips of vessels though it should be realized that they do not necessarily reflect the functional characteristics of the smooth muscle of the resistance vessels which is the vascular section of interest. Most studies of vascular strips have been performed on animals with various types of secondary hypertension (e.g. Redleaf and Tobian 1958 Mallov 1959 Gordon and Nogueira 1962). Vascular strips from rats with primary hypertension (SHR) seem to display unchanged or decreased responses to noradrenaline (Spector et al 1969 Hallböök, Lundgren and Weiss 1971 Mossingham and Shevde 1971 Shibata Kurahashi and Kuchi 1973). Ettinger, Selbel and Riecher (1970) studied the reactivity to noradrenaline of isolated intact small arteries from essential hypertensive man. They concluded that the arteries of hypertensive subjects responded significantly more than those of normal subjects. However quantitative hemodynamic investigations in man where the true resistance vessels rather than conduit arteries are studied (see below) are hardly in agreement with these findings (Sivertsson 1970).

4 Increased myogenic tone might theoretically be a reasonable explanation but it has hardly been discussed in this context simply because the interest has been concentrated on extrinsic excitatory influences on the vascular effector cells (Freis 1960).

5 Changes in ion composition might lead to increased smooth muscle tone or reactivity (Friedman, Friedman and Nakashima 1957 Jones 1973) and have been suggested to be of importance for the initiation of hypertension (Jones 1973). In the latter study performed on young spontaneously hypertensive rats the blood pressure was already considerably increased when the measurements of ionic composition and shifts were done. Further investigations are needed to settle this question (Freis 1960 Nagaoaka, Kikuchi and Aramaki 1970).

However in the established or advanced phases such mechanisms might contribute to the increased resistance (Pickering 1968).

Decreased internal radius due to morphological changes

The presence of morphological changes in the systemic vascular bed of patients with high blood pressure is well known. When Bright in 1836 described a state with albuminuria, dropsy and hypertrophy of the heart. Indirect blood pressure measurements were not yet known and the morphological aspects of hypertension therefore attracted the main attention. Already in 1868 Johnson reported hypertrophy of the arteriolar walls in the kidney, intestine, skeletal muscle, placenta and skin in Bright's disease. Gull and Sutton (1872) considered the vascular changes as the primary and essential condition in hypertension. Mahomed (1881) who was the first to systematically use an indirect method for blood pressure estimations, concluded that there are three stages in Bright's disease; first a functional stage where only pressure is increased, second a chronic stage with organic cardiovascular and renal changes and third a terminal stage with renal failure. Turnbull (1915) stated that the media hypertrophy is constantly associated with persistent elevations of blood pressure and that the degree of hypertrophy in the heart and arteries is so closely related that it is possible to form an approximate estimation of the increase in the weight of the heart from examination of the arteries. Morphological measurements of media hypertrophy and wall/lumen ratio were also performed by e.g. Kernohan, Anderson and Keith (1929), Moritz and Oldt (1937), Sommers, Reiman and Smithwick (1958) and Giese (1966).

Nevertheless, the hemodynamic consequences of such obvious changes in the structural design of the resistance vessels were to a great deal neglected until the middle of this century. One of the reasons for this was the observation that the resistance vessels of hypertensive subjects also dilate readily in response to vasodilator agents, which led to the erroneous conclusion that morphological changes were of little functional relevance. Furthermore, it was not possible until lately to make exact quantitative measurements of the inner radius and the wall/lumen ratio in well defined states of activity and distension of minute vessels, constituting the main site of the precapillary resistance. Not until it was shown that resistance was raised even during maximal dilatation and the hemodynamic consequences of such changes were considered (Folkow 1956, Folkow, G. Imby and Thulesius 1958) was attention drawn to the functional aspects of the structural changes of the resistance vessels. Thus Short and coworkers (Short and Thomson 1959, Short 1966) demonstrated by morphological measurements an increased wall/lumen ratio with reduced lumina in arterioles from patients with high blood pressure but they preferred to denote the changes as vascular contracture though the hemodynamic consequences would be the same as those outlined by Folkow (1956).

However the so far most extensive and precise morphological estimation of the wall/lumen ratio of arteries and arterioles in hypertension has been performed by Suwa's group (Furuyama 1962, Suwa and Takahashi 1971). The length of the internal elastic membrane was measured by attaching a thin thread on the magnified photomicrograph of an arterial cross section from the histological slide and it was used as an estimate of the internal circumference. The surface area of the muscular coat was then planimetrically determined allowing an estimation of the relation between internal radius and media thickness. A close correlation was found between the level of blood pressure and the degree of media hypertrophy. Thus the most extensive hypertrophic changes were observed in arteries and larger resistance vessels while they were gradually decreasing towards the capillary level. It was therefore concluded that the major resistance to flow was offered by larger and medium-sized resistance vessels while the smallest precapillary vessels were more or less protected from the pressure rise.

HEMODYNAMIC CONSEQUENCES OF VASCULAR MORPHOLOGICAL CHANGES

Experimental background of the present studies

After an initial era of morphological studies and classification the hemodynamics of essential hypertension were gradually brought into focus (cf. Pickering 1968). Prinzmetal and Wilson (1936) and Pickering (1936) found by using plethysmographic methods that regional flow resistance in the forearm was higher in hypertensive than in normotensive subjects during rest as well as during dilatation secondary to nerve blockade, indirect heating or arterial occlusion. At that time structural changes were not considered to cause the increased resistance but that some chemical factor was maintaining a vascular hypertonus. However Grant and Pearson (1938) showed that flows to the forearm, measured without previous arrest of the circulation to the hand are mixed muscle and skin flows. Stead and Kunkel (1940) who were aware of this observed no difference between hypertensive and normotensive patients in blood flow of forearm, hand and foot after dilatation and discussed both structural and humoral factors as background to the increased resistance in hypertension. Abramson and Fienst (1942) and later Brod (1963) found an increased muscular blood flow in hypertension during rest indicating that during resting conditions the increased resistance is not uniformly distributed. Brod's extensive hemodynamic explorations (1963) suggested the presence

of a specific neurohormonal discharge pattern resembling a mild defence reaction where a great proportion of the cardiac output is distributed to the skeletal muscles (Ellosson et al 1951 Abrahams Hilton and Zbrazyna 1960 Folkow and Neil 1971). In general a great number of investigations suggest that with exception for the muscles the increased resistance in established hypertension is present in most systemic circuits being unusually high in the kidneys (cf Pickering 1968).

In order to further investigate the exact background of the increased resistance more detailed hemodynamic analyses are required covering the entire range of vascular smooth muscle tone from the level of maximal dilatation to that of maximal constriction. Such studies can provide general information not only about the hemodynamic characteristics in hypertension but also about the smooth muscle sensitivity and the design of the resistance vessels. Folkow (1956) and Folkow Grimby and Thulesius (1958) demonstrated that regional flow resistance was raised even at complete relaxation of the vascular smooth muscles in essential hypertension clearly indicating a fundamental change of the structural design of the resistance vessels. This finding in combination with the morphologically demonstrated media hypertrophy led to the theoretical deduction (Folkow 1956) that for any given increase of smooth muscle contraction the hypertensive resistance vessels would display exaggerated luminal reductions. In other words the presence of an increased wall/lumen ratio should have the hemodynamic consequences of an increased flow resistance and increased vascular reactivity without necessitating any hyperreactivity or hypersensitivity of the smooth muscle cells. Conway (1963) Silverston and Olander (1968) and Silverston (1970) confirmed and extended these findings.

In order to further explore from the qualitative and quantitative point of view the increased wall/lumen ratio of the resistance vessels by means of a hemodynamic approach it was necessary to turn to an experimental model such as the spontaneously hypertensive rat SHR (Okamoto and Aoki 1963) allowing more invasive techniques than those possible to use in man.

Theoretical considerations concerning the preparation and methodological approach

The quantitative hemodynamic approach to the problem of the resistance vessels i.e. their luminal dimensions and reactions has the great advantage that resistance to flow varies inversely to the fourth power of the average internal radius. This marked amplification is of great importance since e.g. a 5 per cent reduction in the radius which is almost impossible to detect with direct morphological techniques implies a more than 20 per cent increase in resistance to flow or in

pressure. If flow is constant. Furthermore, by such hemodynamic measurements average values for all the parallel coupled resistance vessels under study are obtained and moreover the relative influence of the consecutive resistance sections can also be considered (Folkow et al 1974). For such reasons paired perfusion experiments (Folkow et al 1970 b) which will be further outlined in Methods were performed on isolated hindquarter preparations of spontaneously hypertensive rats (SHR) as compared to matched normal control rats (NCR). By such paired analyses both rats are exposed to almost identical experimental conditions using the same perfusate, pump system, drug administrations, temperature etc. and thus a variety of accidental disturbances are ruled out. The resistance to constant flow was recorded during maximal dilatation and during graded constriction induced by noradrenaline infusions (NA) up to maximal constriction as ensured by addition of vasopressin and barium ions. For an evaluation of possible differences in wall/lumen ratio and luminal reductions between SHR and NCR such a hemodynamic approach has a number of advantages over morphological measurements. In addition to those mentioned above. First, the resistance vessels can be studied not only when their contractile elements are completely relaxed, but also when they are maximally contracted against a known load and when they are kept at comparable intermediate levels of activity. This allows recordings of the complete dose-response relationships in the form of resistance curves. Second, at known levels of smooth muscle activity the vessels can be exposed to predetermined changes in transmural pressure, hence allowing precise calculations of wall distensibility from the imposed changes in pressure and flow. Third, a changed smooth muscle sensitivity to e.g. constrictor agents will, if it is the only difference between SHR and NCR, become evident as parallel shifts along the abscissa of the resistance curve and thereby allow a distinction between increased vascular reactivity secondary to increased smooth muscle sensitivity or to an increased wall/lumen ratio (Folkow et al 1970 b).

Furthermore, by utilizing the constant flow technique, drug concentrations will be kept constant even when adjustments in vascular dimensions take place. This technique also allows measurements of the maximal contractile strength of the resistance vessels when they contract against a gradually increasing transmural pressure until an equilibrium is reached between the transmural pressure and the maximal efforts of the vessels. Unless there are any qualitative changes in the vascular walls this must closely reflect the bulk of contractile tissue in relation to the lumen.

Fig 1 (from Folkow et al 1970 b) right part illustrates the theoretically calculated resistance curves for a hypothetical hyper

COMPLEX EXPERIMENTAL RESULTS

PRESSURE PROPORTIONAL TO FLOW RESISTANCE



HYPOTHETICAL RESISTANCE VESSELS

RESISTANCE PROPORTIONAL TO PRESSURE

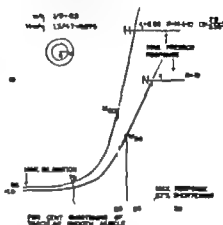


Fig 1 The left part shows the average resistance curves for SHR and NCR based on the results of 15 paired experiments. The right part shows the mathematically deduced resistance curves for two hypothetical resistance vessels H and N where H differs from N only in the respect that its media thickness is increased 30 per cent encroaching upon its lumen even at maximal dilatation. Note the striking similarities between the relationships of the two sets of resistance curves with respect to 1) Resistance at maximal dilatation 2) Threshold (Th) 3) Steepness of the curves 4) 50 per cent of the maximal pressor response (M_{50}) and 5) Maximal pressor response (Folkow et al 1970 b)

tensive vessel H and a "normotensive" vessel N. The curves are constructed according to a number of assumptions:

- 1) The hypertensive vessel H has a 30 per cent thicker smooth muscle sheath than that of the normotensive vessel N.
- 2) Outer dimensions at maximal dilatation are assumed to be the same.

for the two vessels

3 The ratio between wall thickness and internal radius of the normal vessel N is set at 1:5 at maximal dilatation for normally distended completely relaxed arterioles (van Citters 1966) and this ratio will be 1:3.47 for the hypertensive vessel H according to assumption 1. This increased wall/lumen ratio of the hypertensive vessel H has according to the law of Poiseuille the consequence that the resistance at maximal dilatation is 25 per cent higher in H than in N. The thicker wall also implies a greater stiffness.

4 The cross-sectional area of the vessel wall is considered to be constant when the vessels constrict, which is reasonable in the acute situation and is also supported by direct measurements on normal vessels (Baex 1969).

5 It is further assumed that constriction is normally initiated from the outermost muscle sheath, which is true for contractions initiated by vasoconstrictor fibres since their neuroeffector junctions make contact only with the adventitial muscle surface (cf. Ljung 1970). This assumption seems justifiable also when the resistance vessels are exposed to exogenous noradrenaline (NA), since a recent study of the portal vein of the rat (Johansson et al. 1970) suggests that the great majority of the alpha receptors are located in or close to the neuroeffector junctions. For the resistance vessels this would imply that an adrenergically induced constriction displaces the inner tissue layers towards the lumen exaggerating the luminal reduction for a given smooth muscle shortening largely in proportion to the increase in wall/lumen ratio.

The abscissa in the hypothetical diagram of Fig. 1 is given as the percentage decrease of smooth muscle length in a scale derived from the relationship between the logarithm of the noradrenaline dose and the smooth muscle shortening of an isolated aortic strip to make the scale comparable with the effects upon pressure (or resistance) induced by known NA concentrations in the perfused vascular beds. The relationship between NA concentration and smooth muscle shortening forms an S-shaped curve when a strip of smooth muscle is used and such a curve for aortic strips was utilized for transforming NA concentrations into actual shortenings of the smooth muscles. By such a procedure the proportions between consecutive 5 per cent shortenings of the contractile elements of the hypothetical vessels H and N were made more directly comparable to actual shortenings of the vascular smooth muscles caused by the different NA doses in the experiments (for details see Folkow et al. 1970 b). It was arbitrarily assumed that N could maximally contract at constant flow to such an extent that the perfusion pressure was 10 times that of maximal dilatation. Since H had 30 per cent more of contractile wall mass, it would then increase pressure 30 per cent more

and with an adjustment according to Laplace's law a 40 per cent higher maximal pressor response was obtained for H than for N. In this way the entire theoretical resistance curve could be deduced for both H and N.

Experimentally obtained resistance curves (dose/response curves for noradrenaline infusions) from the level of maximal dilatation to maximal constriction can like the mentioned theoretically calculated resistance curves be described by five key points: 1) Resistance at maximal dilatation 2) NA threshold (defined as the NA concentration required to obtain a 25 per cent increase in resistance above that at maximal dilatation) 3) Steepness of the curve 4) M_{50} defined as the NA concentration required for eliciting 50 per cent of the maximal response (for comparisons with ED_{50} for conventional dose/response curves on muscle strips) 5) Maximal pressor response which is defined as the highest pressure level that can be reached with supramaximal NA concentrations and additional bolus doses of vasopressin and/or $BaCl_2$ during constant flow conditions.

A comparison between the experimentally obtained resistance curves and the hypothetically deduced curves (Fig. 1) shows striking similarities with respect to the relationships between the hypertensive and the normotensive curves concerning all five key points mentioned above:

- 1 The flow resistance at maximal dilatation is increased to largely the same extent in H and SHR as compared to N and NCR.
- 2 The NA thresholds are identical for H, SHR, N and NCR.
- 3 The curves for H and SHR are proportionally steeper than for N and NCR respectively.
- 4 M_{50} for both H and SHR are displaced to the left of N and NCR respectively despite equal NA thresholds. This is in H the result of an increased wall/lumen ratio and the same explanation is therefore strongly suggested for SHR.
- 5 The maximal pressor responses are proportionally raised for both H and SHR compared with N and NCR respectively.

In other words: In all respects the relationships between the characteristics of the SHR/NCR curves closely mimic those of the H/N ones, even quantitatively. Thus based on the assumptions given above the interpretation of these characteristics are that a) the increased resistance at maximal dilatation is an expression for the reduced luminal width of the resistance vessels in SHR, b) the increased steepness of the resistance curve with the displacement to the left of M_{50} at identical NA thresholds, is an expression for the raised wall/lumen ratio and c) the higher maximal contractile response is an expression for the increased amount of smooth muscle tissue in the vessel wall of SHR.

Concerning the influences of other types of vascular disturbances structural or functional a thickening of only noncontractile elements such as water logging (Tobian and Binion 1952 Tobian 1972) collagen elements or intimal thickening could explain all the differences except the increased maximal pressor response which calls for an increased amount of contractile tissue. A concentric reduction of the luminal size of the resistance vessels with an unchanged wall/lumen ratio or a rarification of the resistance vessels could explain the increased resistance at maximal dilatation and the unchanged NA threshold but not the other characteristics of the resistance curve. An increased sensitivity to the vasoconstrictor agents would cause a parallel shift to the left of the curve and would not be able to explain any of the changed hemodynamic characteristics in SHR. Lastly if one assumes that morphologically unchanged smooth muscles have for some reason become stronger the increased steepness and maximal pressor response might be explained but not the raised resistance at maximal dilatation or the displaced M50. In fact one would have to combine several of these other hypothetical factors to explain the changed SHR curve and at the same time deny any hemodynamic influence of media thickening, for which there is by now an overwhelming documentation.

The above mentioned hemodynamic characteristics seen in SHR (Folkow et al 1970 a, b) and in essential hypertension in man (Silverstson 1970) have also been confirmed by Haessler and Finch (1972). These characteristics can thus when considered altogether only be explained by an increased wall/lumen ratio where the lumen is reduced even at maximal dilatation and where the wall thickening is at least to a large extent the result of an increased amount of smooth muscle tissue. Such a hypertrophic change in structural design of the resistance vessels will result in a raised resistance level even at normal smooth muscle activity and also allow for an increased range of dilatation - constriction (vascular hyperreactivity) due to the increased wall/lumen ratio. In this way the well known increased resistance in primary hypertension may be explained without necessitating any increased smooth muscle tone as a result of nervous, bloodborne or local excitatory influences. The more closely balanced the extent of the structural change is to the pressure rise the less is needed in enhanced smooth muscle activity to explain how the high pressure is maintained in hypertension. The problem of the increased vascular reactivity has been studied also from other approaches. Thus experiments on aortic strips and portal vein abolishing the influences of the wall/lumen ratio have shown that there is no increase in sensitivity to NA in SHR as compared to NCR (Hallback, Lundgren and Weiss 1971, Shibata, Kurahashi and Kuchi 1973). Furthermore it has recently been shown that the resistance

vessels of SHR are considerably less distensible than those of NCR both during maximal dilatation and during stable levels of smooth muscle tone (Hallböök, Lundgren and Weist 1974). These findings also strongly suggest an increased media thickness provided that no gross quality changes such as collagen etc. of the wall components have also taken place. A decreased distensibility of conduit arteries from hypertensive animals as well as from hypertensive man has earlier been shown (Feigl, Peterson and Jones 1963, Greene et al. 1966, Aars 1968).

More detailed analyses of the hindquarter vascular beds of SHR and NCR utilizing the isogravimetric technique have further confirmed the findings of hypertrophic vascular changes in SHR showing that they are solely confined to the precapillary resistance vessels. The capillary section is largely unchanged and the SHR postcapillary resistance section displays if anything a slightly lower resistance at maximal dilatation and reduced NA responses compared with NCR (Folkow et al. 1974).

Aim of the present investigation

The present series of investigations was designed to study aspects of the functional mechanisms behind the morphological changes of the resistance vessels and the associated changes in hemodynamics found both in hypertensive man and in SHR. From studies on cat hindlimb (Folkow and Silverstein 1968) and in renal hypertensive rats (Lundgren et al. 1974, Lundgren 1974a) it seems as if the hypertrophy of the precapillary resistance vessels could be considered as an exponent of a normal regional adaptation of the vascular bed to changes in pressure load regardless of the background of the increased pressure. This by no means denies that at more severe and sustained pressure increases also degenerative and even lesional elements will supervene as is well known from pathological studies in man as well as SHR (Pickering 1968, Okamoto 1972, Ooshima, Yamori and Okamoto 1972).

In order to estimate the influence of the regional transmural pressure per se on the development of the hypertrophic vascular changes in primary hypertension 3 week old SHR were subject to aortic ligation below the renal arteries i.e. at an age when hypertensive vascular changes are not yet discernable (paper I). To compare the influence of such an extensive hypotension on already established vascular changes similar aortic ligations were performed in adult SHR (paper II).

Furthermore the influence of the sympathetic nervous system on

a possible triggering mechanism by way of its pressure enhancing effect was studied by means of early immunosympathectomy (paper III). As mentioned there is no clear evidence of continuous sympathetic nerve hyperactivity in primary hypertension in man (cf Pickering 1968 Wallin Hogborth and Dellus 1973) but intermittent increases of sympathetic activity may well serve the same purpose in the form of e.g. centrally integrated defence reactions to environmental stimuli (cf Folkow 1960 Brod 1963 Charvat Dell and Folkow 1964 Folkow and Rubinstein 1966 Pickering 1968 Julius Pascual and London 1971). Recent findings of increased plasma catecholamines in patients with essential hypertension (Engelman Partnoy and Sjoerdsma 1970 De-Quattro and Chan 1972) may further support this hypothesis. A long series of studies suggest an increased activity of the sympathetic neuro-hormonal system in SHR (cf Okamoto 1969 1972 Iriuchijima 1973) as a genetically linked predisposing influence: evident also from the observation of exaggerated defence reactions to alerting stimuli (Folkow Hallböck and Weiss 1973 Hallböck and Folkow 1974). This type of autonomic discharge usually implies a more or less raised cardiac output a great proportion of which is distributed to the skeletal muscles. A cardiovascular pattern of this general organisation is seen in "border-line" hypertension in man (Brod 1963 Julius Pascual and London 1971) and also in young SHR (Pfeffer and Frohlich 1973). - Against such a background it was considered of interest to interfere with the β -mediated adrenergic influences in young prehypertensive SHR and in adult SHR with established hypertension (paper IV).

Finally blood pressure was kept at largely normotensive levels in young adult and very old SHR by prolonged treatment with hypotensive drugs used also in man. The effects on cardiovascular structural changes on pressure development after cessation of therapy and on survival rate were investigated (paper V). The influences of age sex and duration of hypertension on the effects of the therapy were also evaluated.

Thus the present series of investigations was designed to study the importance of several mainly functional influences concerning the development possible prevention and regression of high blood pressure hemodynamic characteristics and design of the resistance vessels.

METHODOLOGICAL PRINCIPLES

General experimental procedures

Choice of experimental model

Throughout the present studies spontaneously hypertensive rats (SHR) of the Wistar strain have been used (Okamoto and Aoki 1963) originally obtained by selective breeding from a couple of Kyoto Wistar rats which showed a higher than average blood pressure. In 1968 a small SHR colony came to this laboratory and breeding has continued locally though not consistently between brother and sister to reduce the risk of "passenger" phenomena not relevant for the disorder to be studied.

Genetical analyses of SHR suggest a polygenic mechanism involving a relatively small number of major genetic components (cf Okamoto 1972). A polygenic background seems to be present also in man (cf Pickering 1968, Mall 1971) and since SHR display practically all of the known characteristics of human essential hypertension these rats seem to represent the closest animal model available (Okamoto 1972). Furthermore their homogeneity and the easy control of treatment and environmental conditions throughout a relatively short lifespan imply great advantages.

Anesthesia

Nembutal[®] 4-5 mg/100 g bodyweight was used as anesthesia during the preparation of the hindquarters. In studies I, II and III measurements of arterial pressure were performed during Nembutal[®] anesthesia. In studies I and II ligation of the aorta was performed under ether anesthesia which was also used during the cannulation of the caudal artery after which the rats were allowed to regain full consciousness (papers IV and V).

Measurements of arterial pressure

Indirect measurements. Due to the size and constitution of the rat it is relatively difficult to get adequate measurements of the casual blood pressure and even more difficult to estimate the 24-hour variation and average. Various indirect procedures are described in the literature (e.g. Byrom and Wilson 1938, Friedman and Freed 1969) but as Burag, McCubbi and Page (1971) recently stated "It is often difficult to get a reliable correlation with direct measurements. Others claim to obtain values well correlated with directly measured pressures. If

meticulous care is taken to assure adequate pulsations using an appropriate sensing device (e.g. Pfeffer, Pfeffer and Frohlich 1971)

In the present studies indirect pressure measurements were used only to follow the average effects of prolonged treatment within a group thus evaluating the adequacy of the dosage. The method used was based on a colorimetric principle. The whole rat (or only the tail in paper III) was thus warmed for about 10 minutes at 43-45°C and when the tail became pink. It was blanched by application of a suprasystolic pressure inside a plexiglass cylinder surrounding the tail. Then a cuff was inflated at the base of the tail to suprasystolic pressures. The blanched tail was taken out of the cylinder and the cuff pressure was slowly decreased until the first signs of a skin flush appeared. The pressure at which the skin flush appeared was defined as the arterial pressure. Several modifications of this method and of the more conventional plethysmographic (Byram and Wilson 1938) or microphonic (Friedman and Freed 1969) methods were also tried. However the above described procedure fulfilled the needs also for immunosympathectomized rats in which indirect neurogenic vasodilatation in the tail is eliminated. It was rather easy to handle and it was about as reliable as the other indirect methods in the hands of the same trained technician. Moreover it was sooner or later in each animal always checked against direct pressure measurements (see below).

Direct measurements. Blood pressure was measured under Nembutal^{UR} anesthesia both in the tail artery and in the carotid artery of the aorta-ligated rats in papers I and II and only in the tail in paper III. As observed in control measurements, Nembutal lowers blood pressure in both normotensive and hypertensive rats with approximately 20-30 mm Hg. However tracheostomy usually raises pressures again to a slightly larger extent and addition of unilateral occlusion of the carotid artery (by the cannulation) usually causes a further rise of 20-30 mm Hg. Despite these shifts in pressure levels the relationship between the NCP and SHR pressures were largely maintained.

The technique to measure blood pressure directly during awake conditions after the tail artery was cannulated during brief ether anesthesia (papers IV and V) proved to be the most reliable method. Control experiments where local anesthesia was applied at the base of the tail to eliminate any pain or irritation showed that if the rat stayed awake for 2-3 hours during fairly familiar resting conditions pressure remained largely the same during this period of time in both hypertensive and normotensive rats. This type of direct measurement can further be repeated several times if one starts from the middle of the tail (papers IV and V) and uses gradually more proximal parts for

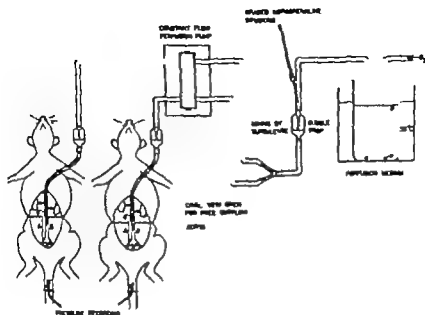


Fig. 2 Schematic illustration of the experimental arrangement during paired hindquarter perfusions.

compulsion

The isolated hindquarter preparation

The hemodynamic analyses were performed on paired isolated hind-quarters according to earlier mentioned principles. An artificial perfusion system was used employing a double Harvard pump and oxygenated Tyrode solution containing 4 per cent Ficoll® (a synthetic polymer of sucrose and epichloro-F-hydrin m.w. approximately 80 000; AB Pharmacia Uppsala Sweden) as plasma substitute. Osmolarity was kept at approximately 300 mOsm/l and viscosity at 1.0-1.2 cP at 37°C. Since the experimental series were performed in the course of several years, different batches of Ficoll® with slight variations in molecular size distribution, hence somewhat influencing viscosity and degree of edema formation had to be used for some of the series. The temperature of the perfusate entering the rats was between 34 and 36°C.

389 However using the paired arrangement as illustrated in Fig 2 the influence of possible changes in g temperature viscosity onto

larity Ficoll and ion contents of the perfusate could be minimized. Further, by the paired perfusion arrangement the weight, age and sex matched rats did not only get the same concentrations of constrictor substance but also the same amounts per body weight whichever the rate of infusion, size of syringe or infusion apparatus used. The preparation of the two rats was also performed strictly in parallel and with identical techniques.

A more pure muscle preparation was used than that described originally (Folkow et al. 1970 b) since measurements of both blood pressure and perfusion pressure were performed in the tail artery and therefore both the tail distal to the cannula and the feet were excluded by tight ligatures.

After pressure measurement in the awake state the animals were heparinized, anesthetized with Nembutal and eviscerated after which the aorta and caval veins were freed for about 1–1.5 cm proximal to the iliac arborization. The hindquarters were isolated by several standardized ligatures around all the tissues, leaving the aorta and caval vein intact. Then the two aortas were rapidly connected via identical polyethylene tubes to the perfusion system; the caval veins were cut wide open for free outflow and perfusion was immediately started at a flow of approximately 10 ml/min \times 100 g. The hearts were subsequently extirpated and divided into the left and right ventricles which were weighed separately. Papaverine, proven to be the most effective vasodilator, was given as repeated 1 mg injections in the common tube to ensure complete relaxation of the vessels. However, during artificial perfusion at such low flow rates the vessels gradually reach virtually complete relaxation even without dilator drugs. In these respects there was never any difference between NCR and SHR; thus inherent vascular tone was not better preserved in SHR.

When no further relaxation could be obtained the vascular bed was considered to be in a maximally relaxed state and flow was changed randomly (5–40 ml/min \times 100 g) while the pressure changes were recorded via the tail artery cannula and a transducer on the Grass Polygraph (Model 7). Flow was then set at 10 ml/min \times 100 g tissue and step-wise noradrenaline (NA) infusion from subthreshold (0.04 μ g/ml perfusate) to supramaximal doses (3 μ g/ml perfusate) was started. Finally to ensure maximal constriction supramaximal doses of vasopressin (10 IU) and BaCl_2 (150 mg) were added as bolus injections in the common tube. Equal distribution of vasoactive agents to the two preparations was ensured by continuous shaking (by means of a motor) of the common bubble trap distal to the site of drug injection.

At the end of the experiments all parts of the rat except the perfused hindquarters were weighed and subtracted from the total body

weight in order to get an exact weight of the perfused tissues without including edema. This is necessary since edema forms on organs during NA infusion and accelerates after the administration of vasopressin and BaCl_2 because these drugs particularly strongly constrict the postcapillary resistance vessels (Folkow et al 1974). Pressure-flow curves during maximal dilatation and resistance curves for the resistance vessel responses were constructed and treated as described in a previous section in which the methodological approach and its advantages were considered. Below the five "key points" of the resistance curves will be discussed from the methodological point of view.

1) Resistance at maximal dilatation reflects the structurally determined average luminal width of the resistance vessels when the smooth muscles are completely relaxed. The question is how to ensure that they are completely relaxed and this was done in various ways: a) by using an artificial perfusion, neurogenic and circulating constrictor influences were eliminated and tissue ion composition made largely equal. This is advantageous when the aim is to study the hemodynamic consequences of a changed resistance vessel design. b) Several potent dilator substances such as nitrites and isoprenaline were tried, but repeated large doses of papaverine proved to be the most effective drug. c) If sufficient amount of time was allowed to pass, vascular tone diminished spontaneously, probably because of factors like the low pressure and low flow rate etc. and papaverine did then not give any further relaxation, even when large doses were given. d) It was furthermore controlled that pressure returned to the initial level after shortlasting increases in flow, i.e. then the vessels were not fully relaxed. Pressure then returned to a lower level than before the pressure rise when a maximally dilated state was ensured. In such ways there were no signs of autoregulation or other active changes in resistance or of any gradually increasing vascular tone.

2) The NA threshold is defined as the NA concentration required to raise resistance 25 per cent above the state of maximal dilatation, which implies roughly a 5 per cent shortening of the contractile elements. The wall/lumen ratio has in this state of maximal constriction very little influence even in the fairly thick-walled resistance vessels. The greatest influence on "threshold" would then be an increased smooth muscle sensitivity, displacing the resistance curve to the left even in its earliest part, which was seen e.g. after irrisympathectomy (paper III).

■ Steepness of the resistance curve is expressed as the tangent of the angle between the steepest part of the curve and the abscissa and is as earlier outlined an exponent for the wall thickness in relation to the lumen during given degrees of smooth muscle shortening regard less of what has caused the wall thickening (e.g. muscle hypertrophy collagen elastin water endothelial thickening etc.) A change only in smooth muscle sensitivity would not affect the curve steepness but rather shift the curve in a parallel manner along the abscissa. When the contraction is initiated from the outer muscle layers the bulk of the muscle tissue together with all other wall elements situated inside the main line of force must be displaced towards the lumen. In papers I and III correction was made for the fact that the SHR curve starts from a higher resistance tending to give a proportionally steeper angle in a coordinate system (Folkow et al 1970 b; see also Sivertson 1970). However in the more complex studies in papers II, IV and V where the main topic was to follow changes in this already known vascular characteristic of SHR no such corrections for the slopes were performed.

The steepness of the resistance curve is a fairly sensitive parameter insofar as it calls for as many points of measurement as possible along the steep part of the curve and a standardized procedure for plotting the curves. However the paired perfusion arrangement is here of the greatest advantage since both recordings occur simultaneously under identical conditions.

4. M_{50} is defined as the concentration of NA eliciting 50 per cent of the maximal NA response. This parameter is not only dependent upon the smooth muscle sensitivity but also on the wall/lumen ratio and the maximal pressor response to NA and is therefore not equivalent to ED_{50} in dose-response curves for muscle strips (Folkow et al 1970 b). M_{50} has only limited relevance in the present studies because several of the therapeutic procedures may have interfered with smooth muscle sensitivity.

5. The maximal pressor response as elicited by supramaximal doses of constrictor substances such as $BaCl_2$ and vasopressin is considered to reflect the bulk of contractile elements in relation to the lumen since an increase in passive wall elements would only slightly increase this parameter (Folkow et al 1970 b). However vasopressin and $BaCl_2$ activate the postcapillary resistance vessels more strongly than NA (Folkow et al 1974) hence rapidly increasing edema formation. In general edema formation was greater in NCR than in SHR tending to give NCR falsely too high maximal pressor responses because of more pronounced increases in tissue pressure. However such interferences will not affect the maximal pressor responses more than about 5 per cent.

as explored in other studies (Folkow et al 1974). The large doses of vasopressin and barium chloride were rather equal in potency and if vasopressin was given first the other agent added very little and vice versa.

Statistical calculations

In papers I and III statistical evaluation of the hemodynamic analyses was performed according to the group comparison t test. The left ventricular heart weights (papers IV and V) were also compared by means of this test.

In papers II, IV and V a pairing design t test was used for statistical evaluation of the difference between the test animal and its paired control. However, for comparison between groups of treated SHR - NCR and untreated SHR - NCR a group comparison t test of the differences was performed (Woolf 1968).

The difference between groups was considered significant at p-values below 0.05.

Special experimental procedures

Aortic ligations

This procedure was performed in young prehypertensive SHR to investigate to what an extent the development of hypertensive vascular changes are dependent upon the local transmural pressure. Aortic ligation was also performed in SHR in the established phase of hypertension to follow the rate and extent of regression of the already present vascular changes. The aorta was totally obstructed by a silk ligature so that the hindquarters were subsequently dependent upon collateral blood supply. Already one day after the operation the rats seemed to be largely unaffected by the operation and postoperative mortality was low. The young rats developed collaterals very rapidly and after 11 weeks hindquarter blood pressure had reached the level of 85-95 mm Hg. Their gain in body weight and also that of their hindquarters was largely normal. Of adult rats only females could be used, presumably because of their better collateral supply via the ovaries. However, the adult female SHR seemed to recover almost as rapidly as young SHR and after two days they were able to walk freely. Immediately after operation local blood pressure was about 25 mm Hg and after 19 weeks about 80 mm Hg in adult SHR. There were no signs of tissue atrophy in the hindquarters.

Attempts were made to obtain a more moderate pressure reduction

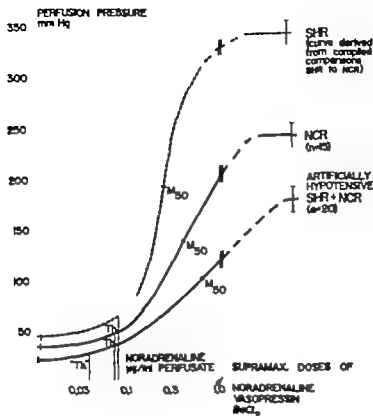


Fig 3 Average resistance curves of constant flow perfused hindquarter vascular beds from aortalgated SHR - NCR and untreated SHR - NCR. Note how the three curves differ in proportion to the blood pressure in the hindquarters of aortalgated SHR: NCR 97 ± 12 and 80 ± 6 mm Hg; pooled into one curve in the NCR hindquarters 145 ± 4 and in the SHR hindquarters 203 ± 8 mm Hg.

maximal pressor response (0.72) but the steepness of the resistance curve (see paper II) mainly reflecting the wall/lumen ratio remained relatively increased (0.89). This may indicate that in more advanced stages of hypertension there are other elements present in the walls of the resistance vessels such as collagen and elastin which display far less regression than does the smooth muscle component (cf Wallinsky 1971, 1972). Young prehypertensive SHR showed on the other hand

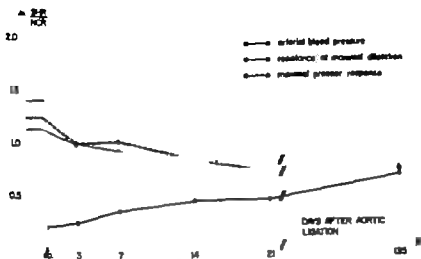


Fig. 4. Diagrammatic illustration of the regional blood pressure drop and the extent of structural vascular adaptation in the hindquarters with time after aortic ligation in adult female SHR. All values are expressed as the ratios for SHR/NCR and for aortaligated SHR/NCR.

complete adaptation also of the steepness of the resistance curves upon pressure reduction indicating a difference in plasticity between young and adult animals in this respect.

Immunosympathectomy

Immunosympathectomy was performed in newborn SHR and NCR to produce an extensive reduction at an early age of the functional trigger influence exerted by the sympathetic nervous system upon the development of high blood pressure and vascular changes. At the age of 13 months when the acute experiments were performed the directly measured blood pressures were for sympathectomized SHR and NCR 139 and 113 mm Hg respectively as compared to 201 and 139 mm Hg for untreated SHR and NCR. These results suggest like those of Clark (1971) that the sympathetic nervous system is of great importance for the development of primary hypertension in rats. However even if the

Furthermore when the blood pressure and vascular bed of untreated NCR were compared with those of treated SHR both having similar resting blood pressures the treated SHR exhibited significantly higher values for curve steepness and maximal pressor response (Fig. 6). Thus even though there was an extensive destruction of cardiovascular adrenergic nervous control in the treated SHR they were able to maintain a largely normal blood pressure. However this was associated with modest hypertensive changes in resistance vessel design which might indicate that SHR have a greater tendency to mesenchymal hypertrophy to a given load. This possibility has also been discussed by Folkow, Grimby and Thuleius (1958) Siverthsson (1970) and Folkow et al (1973).

β -adrenergic receptor antagonists in young and adult SHR

As previously discussed the presence of a neurogenic pattern similar to a mild defence reaction has frequently been observed in early essential hypertension in man as well as in young SHR. Thus in order to interfere pharmacologically with the adrenergic influences on the heart β -adrenergic receptor antagonists were administered in the drinking water (paper IV). Two and a half month old SHR still largely in the prehypertensive phase of hypertension were treated either with a β_1 -receptor antagonist or with propranolol (β_1 and β_2 -receptor antagonist) up to 8 months of age. Adult SHR in the "established" phase were treated with propranolol from 8 until 10 months of age.

When treatment was interrupted at 8 months of age in the first mentioned SHR group their arterial pressure was essentially the same as when treatment was initiated being thus considerably lower than that of untreated adult SHR. The hemodynamic analyses of the hindquarters showed that also the structural design of the resistance vessels was largely the same as when the therapy started i.e. considerably different from the situation in adult untreated SHR (Fig. 7). Thus the resistance at maximal dilatation was at a normal level and the steepness and the maximal pressor response were only slightly increased compared to NCR. There was no difference in these respects between the β_1 - and the combined $\beta_1 + \beta_2$ -blockers. Therefore early treatment with adrenergic β receptor antagonists evidently prevented further development of the hypertensive disease but did not manage to bring the situation to an entirely normal level.

Propranolol treatment in adult SHR with established hypertension did not reduce resting blood pressure although the resistance at maximal dilatation was reduced as was the maximal pressor response in females.

YOUNG SHR TREATED WITH PROPRANOLOL OR H 93/26

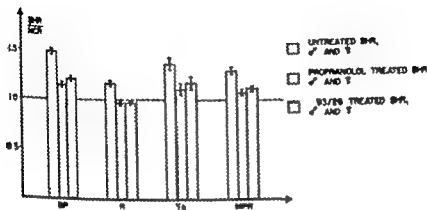


Fig. 7 The ratio \pm SEM between untreated ($n = 26$) propranolol ($n = 17$) or H 93/26 ($n = 18$) treated SHR from age 2.5 to 8 months and their respective NCR concerning blood pressure (BP) resistance at maximal dilatation (R) steepness of the resistance curve (Tg) and maximal pressor response (MPR)

However the main exponent for the wall/lumen ratio i.e. the steepness of the resistance curve was not at all affected in these adult SHR

Long-term hypotensive treatment in young adult and old SHR

It was considered of interest to investigate in more detail the effects of chronic substantial pressure reduction by hypotensive drugs in young adult and old SHR concerning first the development of hypertension after cessation of therapy second the survival rate and third the effects on already established structural changes. A combination of hydralazine and guanethidine was used as treatment

1 Blood pressure after cessation of therapy

SHR were treated from the age of 2.5 months when blood pressure barely raised and when only slight vascular changes are detectable by

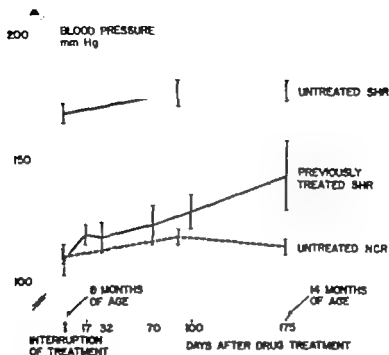


Fig 8 Changes in Intraarterially measured blood pressure \pm SEM of 11 SHR previously treated between 2.5 and 8 months of age as compared to 15 NCR and 15 untreated SHR and followed for 6 months after treatment was interrupted

Hemodynamic analyses The treatment was interrupted after 5.5 months when direct pressure measurements were performed. During the subsequent months pressure increased quite slowly reaching 145 mm Hg only after several months (Fig 8) and was throughout far lower than in untreated SHR.

2 Survival rate

Survival rate after the age of 12 months was considerably improved in SHR previously subject to treatment between 2.5 and 8 months of age as compared to untreated SHR or SHR treated only from the age of 12 months (Fig 9). There was no difference in survival rate between

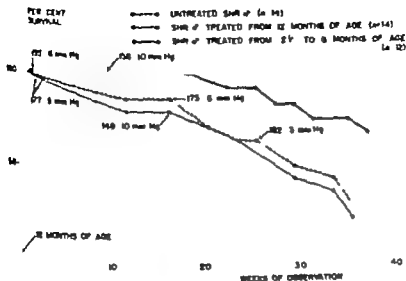


Fig 9 The survival rate of untreated male SHR of male SHR treated from 12 months of age and of male SHR previously treated between 2.5 and 8 months of age as followed between the age of 12 and 21 months. Values for mean blood pressure (\pm SEM) are also presented

as previously treated with guanethidine-hydralazine and those previously treated with propranolol

3 Effects upon the structural vascular changes

Adult SHR - The hindquarters of SHR treated from the age of 8 months were subject to hemodynamic analyses after 5 and 10 weeks of treatment and also 2 weeks after interruption of 20 weeks of treatment. The results are presented in Fig 10 as ratios between treated and untreated SHR and their normotensive controls. After 5 weeks of treatment when blood pressure was normalized the resistance at maximal dilatation and the maximal contractile strength of the resistance vessels were reduced towards normal levels. However the steepness of the resistance curves reflecting the wall/lumen ratio remained increased. No further alteration in these respects was obtained after 10 weeks of treatment. However an analysis of differences between the sexes suggested a more pronounced regression of the structural vascular changes

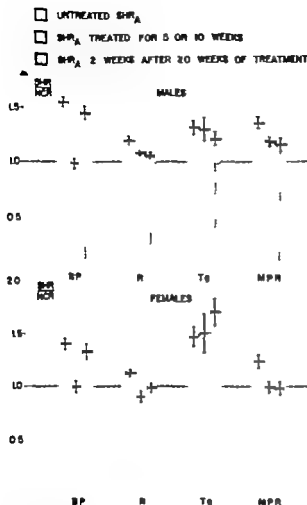


Fig 10 The ratio \pm SEM between untreated (14 males and 12 females) treated (9 males and 8 females) and previously treated (7 males and 4 females) SHR and their respective NCR concerning blood pressure (BP) resistance at maximal dilatation (R) steepness of the resistance curves (Tg) and maximal pressor response (MPR)

in females than in males. Similar results regarding sex differences have been reported by Wolinsky (1971, 1973) on the thoracic aorta of rats earlier exposed to renal hypertension.

In the rats treated for 20 weeks blood pressure was followed for two weeks after cessation of treatment. During that time pressure rose fairly rapidly reaching levels only 15-20 mm Hg below those in untreated SHR controls. The hindquarter vascular bed still displayed largely the same characteristics (Fig. 10) as during treatment i.e. an unchanged increase in the steepness of the resistance curves suggesting the presence of a maintained increase in wall/lumen ratio but combined with lowered resistance at maximal dilatation and lowered maximal pressor response.

Thus compared to SHR treated from young age, adult SHR treated only when their hypertension was already established showed an entirely different time course in the pressure rise upon interruption of treatment. Furthermore when considering the speed at which all signs of cardiovascular hypertrophy were abolished after reversal of renal hypertension in 10 week old rats (Lundgren 1974 a) it is evident that the age of the animal and the duration of the hypertensive state are both very important factors with respect to the possibilities of pressure normalization and regression of hypertensive structural changes (Wolinsky 1972).

It is probable that the signs of a remaining increase in wall/lumen ratio of the resistance vessels in adult SHR reflect the presence of enhanced amounts of collagen and other more degenerative wall changes which persist in spite of pressure reductions. If an increased wall/lumen ratio is maintained it may be expected that resistance returns to supranormal levels when a normal smooth muscle tone is restored after cessation of therapy. Also the bigger arteries would tend to remain stiffer (cf Wolinsky 1971, 1972) which would prevent the baroreceptors from effective resetting back to normotensive pressure levels (cf Aars 1969).

Old SHR The older the organism and the higher the blood pressure the more difficult it is to normalize blood pressure and cardiovascular design. This was illustrated in a group of old (32 months) severely hypertensive SHR that were treated for 13 weeks. At this age SHR display gross cardiovascular as well as renal lesions (see Okamoto 1972, Oshima, Yamori and Okamoto 1972). Treatment caused only a modest pressure reduction (to 150 mm Hg) and there was only a moderate regression in the vascular changes. Particularly no significant change was seen in the parameter reflecting the wall/lumen ratio.

Effects of treatment on cardiac hypertrophy

The influence on cardiac hypertrophy of the pharmacological treatments (papers IV and V) is minute in SHR. This might at least in part be

characteristics as earlier shown in man (cf Folkow et al 1973) i.e. an increased resistance at maximal dilatation in most vascular beds and increased resistance responses to graded infusions of noradrenaline (NA) though without signs of any increased smooth muscle sensitivity to NA. Furthermore the isolated SHR hindquarters displayed increased maximal pressor responses reflecting an enhanced contractile strength of the media. These hemodynamic characteristics were theoretically analysed and interpreted as exponents of a changed design of the resistance vessels i.e. an increased wall/lumen ratio associated with narrowed lumina. The increased wall thickness is at least in early phases predominantly constituted by an increased bulk of contractile tissue. Recent studies (Folkow et al 1974) have revealed that this change is strictly confined to the precapillary resistance vessels.

The present investigations were undertaken to analyse why when and how rapidly such changes take place in the course of hypertensive disease and how they can be either prevented or brought into regression once they are already established. By aortic ligation an extensive reduction in regional pressure was produced both in young and adult SHR. In young SHR the development of the characteristic vascular changes was thereby entirely prevented. However in adult SHR with already established hypertension, the extensive local pressure reduction rapidly diminished the vascular alterations though less completely than in young SHR. Thus signs of a partly maintained increase of wall thickness perhaps reflecting the presence of an increased collagen component in this later phase of hypertension was demonstrated (see also below). These results in combination with the findings in hypotensive cat hindlimb (Folkow and Siverstam 1968) and in young renal hypertensive rats (Lundgren et al 1974, Lundgren 1974 a) where the adaptive structural changes rapidly follow the blood pressure changes in both directions suggest that both heart and resistance vessels readily respond with reversible hypertrophy to regional or general increases in pressure load whatever is the initiating mechanism behind pressure change. Thus the structural changes observed in primary hypertension seem to be a qualitatively normal adaptation to a functionally increased pressor load.

Neurogenic mechanisms are likely to serve as a triggering influence and early phases of primary hypertension are often characterized by a pressure rise associated with an increased cardiac output and increased flow to the skeletal muscle (e.g. Brod 1963, Julius, Pascual and London 1971, Pfeffer and Fohlich 1973). These characteristics suggest the involvement of a mild defence reaction i.e. the response pattern usually elicited by a variety of alerting stimuli (Folkow and Neil 1971). Studies by Okamoto and his group provide evidence

of an enhanced neurohormonal discharge to the cardiovascular system in SHR (Okamoto 1969 Okamoto 1972 also Iruhijima 1973). In addition alerting stimuli induce increased and more prolonged rises in pressure and heart rate in SHR than in NCR (Folkow Hallböck and Weiss 1973 Hallböck and Folkow 1974). Studies of patients with essential hypertension also seem to suggest such a hyperreactivity (e.g. Kalis et al 1957 Nestel 1969 Lorimer et al 1971).

Since such reaction patterns involve an accentuated neurogenic drive on the heart as an important component chronic treatment with β -adrenergic blockers seemed to be a suitable tool for exploring the importance of this component for the development of established hypertension. However it is hardly possible at present to associate the effects of this long-term intensive treatment with any specific function mediated by β -adrenergic receptors since the drugs used display a diverse mode of action perhaps also affecting central autonomic mechanisms (e.g. Bühler et al 1972 Fitzgerald Wale and Austin 1972 Tarazi and Dustan 1972 Samnerstedt 1974). Whatever the dominant mode of action this type of treatment prevented further development of hypertension and its vascular sequelae when used in young prehypertensive SHR but in the adult SHR the high blood pressure was not at all influenced. These findings suggest that the β -receptor antagonists used have somehow critically interfered with excitatory influences on the cardiovascular system in young SHR thereby also preventing the structural vascular changes which appear to be crucial for the development of established primary hypertension. On the other hand preventive propranolol treatment of this type did not have any effect on the development of renal hypertension suggesting that the trigger mechanisms in renal hypertension are of a different nature (Lundgren 1974 b).

The importance of the sympathetic nervous system for the development of primary hypertension was further confirmed by studies on transsympathectomy in newborn SHR causing an extensive reduction of adrenergic excitatory influences in general. This treatment also prevented the development of hypertension although the treated SHR remained at a higher pressure level than similarly treated NCR. Despite the fact that the treated SHR displayed the same blood pressure as untreated NCR there were still significant structural changes in their vascular beds. Possibly these changes reflect that SHR are genetically somewhat more prone to respond to a given pressure load with cardiovascular hypertrophy. The considerable cardiac hypertrophy seen in SHR treated with β -receptor antagonists from an early age may point in this direction. In this connection it is of interest that a prevalence for a genetically determined mesomorphic body build

has been observed in human essential hypertension (Robinson and Brucer 1940). This finding probably implies that the tissues in general including the cardiovascular system tend to respond somewhat more than normally with hypertrophy when exposed to an increased functional load (Folkow 1956, Folkow, Grimby and Thulesius 1958, Folkow et al 1973). Comparisons between renal hypertensive rats with long-standing hypertension and SHR may also point in this direction (Folkow et al 1973, Lundgren et al 1974).

The procedures which brought SHR closest to similarly treated NCR with respect to blood pressure was the treatment with guanethidine and hydralazine i.e. a general peripheral sympathetic blockade in association with a depression of precapillary myogenic tone. Also in adult SHR with not too far advanced hypertensive changes this type of treatment caused efficient pressure reduction and considerable regression of the changes of the resistance vessels in a relatively short period of time. Even in advanced hypertension some reduction in pressure and alteration of the structural vascular changes could be obtained. However in both these phases of hypertension the increased left ventricular weight as well as the increased wall/lumen ratio of the resistance vessels seemed to remain largely unchanged. These findings probably reflect the presence of structural changes involving e.g. collagen invasion which is far more persistent than uncomplicated muscle hypertrophy. Several investigations, mainly dealing with larger arteries suggest that along with muscle hypertrophy there is also an increase of fibrous tissue elements which may develop more slowly but may also be less reversible (Rodbard 1970, Wolinsky 1970, 1971, 1972). According to Olsen (1971) development of fibrous elements in the vascular walls can be traced as early as within a few days after acute hypertension. Long duration of the hypertensive state thus tends to introduce more or less irreversible structural changes in the cardiovascular system as well as gross lesions in e.g. kidneys (Okamoto 1969) which in turn may cause an additional element of renovascular hypertension. Once such more persistent changes in the resistance vessel wall occur it is far more difficult to normalize the cardiovascular situation. This is to be compared with the total regression of the vascular changes seen after reversal of shortlasting renal hypertension in young rats (Lundgren 1974 a).

Sex also seems to be of importance in these respects since the females were more responsive to treatment than the males. This is in agreement with the findings of Wolinsky (1972, 1973) suggesting a depressive influence of female sex hormones upon the development of collagen and elastin in the aorta during renal hypertension.

When treatment was interrupted in SHR exposed to preventive

treatment from youth into adult age blood pressure rose quite slowly contrary to the situation when treatment had been started only in adult age. In the latter case blood pressure increased rapidly upon cessation of treatment. These findings suggest that the response of the tissue to the functional load is more vivid and efficient at an early age since the development of hypertension is much slower and less severe if the neurogenic impact is delayed until adult age. Frels et al (1972) made similar observations on SHR concerning development of blood pressure and vascular lesions after cessation of prolonged pharmacological treatment.

In the present study it seems possible to obtain an evaluation of the influence of connective tissue formation in the resistance vessels on the course of hypertension and on the possibilities to induce effective regression of the disease by therapy. The maintained increase in wall/lumen ratio in adult treated SHR might explain why it is difficult to maintain a lowered blood pressure if treatment is interrupted. If the wall/lumen ratio remains increased a return of normal smooth muscle tone after cessation of therapy would tend to again result in a raised resistance. Furthermore changes in wall structure maintaining an increased wall stiffness at the site of the baroreceptors would interfere with their resetting to a lower level during the treatment (cf Aars 1969). Once substantial collagen invasion has occurred with in the cardiovascular high-pressure sections the homeostatic reflex control would tend to restore the hypertensive situation, despite the therapeutic efforts.

Summarizing these considerations it can be stated first and foremost that the changes in cardiovascular design are secondary to functional increases in average pressure, whether intermittent or continuous and whichever the background. This by no means denies that the extent of these changes for a given pressure increase can be moderated by other influences. If the vascular bed can be protected from such a pressure load from young age these changes are largely prevented. On the other hand the older the individual gets and the longer the changes have been present the more difficult it is to induce their regression because the early smooth muscle hypertrophy probably becomes complicated by slower but more persistent connective tissue changes and degenerative phenomena. Females are in a better situation than males in these respects.

If hypotensive treatment is given from young age over a considerable period of time and is thereafter interrupted there will only be a quite slow and modest progression of blood pressure over a long period of time. Furthermore such a period of early treatment considerably increases survival rate after 12 months of age even when

compared with SHR which are under permanent treatment but only from this adult age. It therefore seems evident from these studies that for any kind of hypertension the time in life when the functional excitatory influences are changed is crucial for the further development of the disease. The tissue response to the functional pressure load seems to be greatest at a young age whereas in older individuals the tissues react more slowly. Therefore the morphological cardiovascular adjustments which are so important for the establishment and maintenance of a truly hypertensive state are likely to develop more slowly with age.

The inherent hyperreactivity concerning qualitatively normal autonomic mechanisms conveying neurogenic pressor responses to alerting stimuli seen in SHR and perhaps also present in essential hypertension in man, might serve as an efficient trigger mechanism. Further since sustained increases in blood pressure can be induced by tonic hypothalamic stimulation (Folkow and Rubinstein 1966) and by stressful environment (Henry, Meehan and Stephens 1967; Gutman and Benson 1971) even in otherwise normotensive individuals. It is evident that exogenous influences may further enhance the functional load upon the cardiovascular system and thus be of considerable importance for the development of an established hypertensive state. Structural adaptation in response to the increased functional load gradually occurs and finally in the established phase it may to a great extent be responsible for the increased resistance and blood pressure. However it might also be so that other genetically linked predisposing elements than those of neurogenic origin have to contribute in case a more considerable and persistent pressure rise should be established. One such additional element in primary hypertension may simply be a somewhat enhanced tendency of mesenchymal tissues to respond with hypertrophy when exposed to increased load. If so primary hypertension might be characterized by quantitative rather than qualitative differences from the normal individual with respect to both these elements i.e. a increased central neurogenic reactivity and an increased tendency towards tissue hypertrophy.

SUMMARY AND CONCLUSIONS

The present series of experiments was performed to explore the relationships between functional and structural factors for the establishment of the raised resistance in primary hypertension as studied in spontaneously hypertensive rats (SHR) by means of a quantitative hemodynamic approach. Earlier hemodynamic studies in man and SHR have revealed an increase in wall thickness in the systemic resistance vessels at least in early phases mainly consisting of smooth muscle hypertrophy with encroachment upon the lumen in most vascular circuits even during maximal dilatation.

1 Regional hypotension was performed by means of aortic ligation in young prehypertensive SHR and in adult SHR with established hypertension as well as in matched normotensive control rats (NCR). This procedure prevented respectively reduced the structural changes characteristic of hypertensive resistance vessels. It was concluded that an increased transmural pressure is the main prerequisite for the development and maintenance of these structural vascular changes and that they are exponents of a per se normal adaptation in design present also in normotensive vascular beds.

2 Interference with the sympathetic influences upon the cardiovascular system in SHR and NCR was performed by means of a) adrenergic β -receptor antagonists and b) immunosympathectomy.

a) Intensive treatment with adrenergic β -receptor antagonists in young SHR largely prevented both the further development of hypertension and the structural cardiovascular changes. However similar treatment of adult SHR did not lower the already raised blood pressure and had only minor effects on the cardiovascular changes. It was concluded that interferences with sympathetic effects mediated by peripheral and perhaps also central β -receptor mechanisms can prevent the further development of hypertension in young animals but can only to a minor degree affect already established hypertension in adult SHR.

b) Immunosympathectomy at birth prevented the development of high blood pressure in SHR thus also indicating an important role of the sympathetic nervous system for the initiation of this type of primary hypertension in rats. However despite the extensive cardiovascular denervation SHR were able to maintain their blood pressure at a normal level although it was higher than in similarly treated NCR. In addition the treated SHR displayed some signs of hypertensive vascular changes compared with untreated NCR but to a reduced extent than in untreated SHR. These results might also indicate that SHR for a given pressure

load display a somewhat increased tendency of cardiovascular hypertrophy compared with NCR

3 The effects of chronic substantial pressure reduction by means of treatment with guanethidine-hydralazine in young adult and old SHR were investigated concerning blood pressure after cessation of therapy survival rate and structural cardiovascular changes

a) It was shown that after cessation in the adult age of treatment which had kept blood pressure at a normal level from early age there was only a slow and modest blood pressure rise over several months. Furthermore the survival rate was considerably improved by such an early period of hypotensive treatment compared with untreated SHR or with SHR treated only from adult age. There was no difference in these respects between rats treated from young age with guanethidine-hydralazine and those treated from young age with propranolol

b) Prolonged guanethidine-hydralazine treatment of adult SHR keeping them at normotensive levels for long periods did not result in full regression of the vascular changes leaving obvious signs of an increased wall/lumen ratio of the resistance vessels. This might reflect the addition of more permanent wall changes in long standing hypertension as a result of e.g. collagen invasion. Probably as a result of such preserved vascular changes in adult SHR blood pressure rapidly returned to levels rather close to those of untreated SHR upon cessation of therapy. Comparing the sexes regression of the vascular changes tended to be more extensive in the females

It was concluded that central neuro-hormonal excitatory mechanisms whether inherent or exogenous in origin seem to be crucial for the initiation of the primary hypertension in SHR and that the cardiovascular structural alterations represent secondary adaptive changes. However these changes seem to be so rapidly developed that they became in time more or less intertwined with the functional excitatory influences. Furthermore it is not impossible that the structural cardiovascular adaptation to a given pressure load may be somewhat more pronounced in extent in SHR. Other general influences such as age sex and duration of the high pressure state are also important both for the establishment of hypertension and for the possibilities of its reversion

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RELATION BETWEEN THE VOLUME OF
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IN FIBRES OF VENTRAL AND DORSAL ROOTS
AND OPTIC NERVE IN NORMAL GUINEA-PIGS
AND REHABILITATED FROGS

An ultrastructural study

BY
J. J. J. J.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 410

From the Neuropathological Laboratory Institute
of Pathology University of Göteborg, Sweden

**RELATION BETWEEN THE NUMBER OF
MYELIN LAMELLAE AND AXON CIRCUMFERENCE
IN FIBRES OF VENTRAL AND DORSAL ROOTS
AND OPTIC NERVE IN NORMAL, UNDERNOURISHED
AND REHABILITATED RATS**

An ultrastructural morphometric study

by

ANDERS SIMA

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I INTRODUCTION

Nutritional deprivation initiated early in life may have deleterious effects on both the central and peripheral nervous system (Bass et al. 1970 Clos and Legrand 1969 1970 Dickerson et al., 1967 Dickerson and Walmsley 1967 Dobbing, 1964 Hedley Whyte 1973 Sima, 1974). Nutritional rehabilitation following periods of undernutrition has revealed irreversible distortions of central and peripheral nervous system, depending on the onset and severity of undernutrition and the onset of refeeding (Dobbing, 1968 1971 Hedley Whyte 1973 Sima 1974 Sima and Sourander 1974). Previous studies have shown that protein-calorie undernutrition instituted early in life causes retardation in nerve fibre growth of both central and peripheral nerve tracts of the rat (Clos and Legrand 1969 1970 Hedley Whyte and Meuser 1971 Hedley Whyte 1973 Sima et al. 1971 Sima, 1974 Sima and Sourander 1974). As demonstrated by Hedley Whyte and Meuser (1971) both axonal growth and deposition of myelin are affected to the same extent in rat sciatic nerve by undernutrition. Recent examinations by Hedley Whyte (1973) have, however, indicated that in the rat sciatic nerve the deposition of myelin is more affected by undernutrition than the axonal growth. Findings from our laboratory have revealed irreversible impairments in total nerve fibre calibre growth in the sensory dorsal root (Sima, 1974) and in the optic nerve (Sima and Sourander 1974) of prenatally undernourished rats, subsequently nutritionally rehabilitated from the time of birth to adult ages. The fibre growth retardation of the motor ventral spinal root was readily recovered by early initiated rehabilitation. Hedley Whyte (1973) claimed that myelination of sciatic nerve fibres in undernourished rats is only partially restored by rehabilitation. A partial restoration of the total fibre diameters of the sciatic nerve was also found by the author (Sima, 1974). This was interpreted as presumably being due to the summation of the in principle different effect of rehabilitation on the fibres of the ventral and dorsal spinal roots both of which merge into the mixed sciatic nerve.

The present study concerns the fibre population of the ventral and dorsal spinal roots of the fifth lumbar segment and of the optic nerve in normal undernourished, and nutritionally rehabilitated rats. The fibres are characterized by their axon circumference axon diameter calculated from the axon cross-sectional area, and by their number of myelin lamellae. The first and the last mentioned parameters used in the current study for classifying myelinated fibres, provide a sensitive procedure for the analysis of the relationship between axonal growth and myelin deposition (Friede and Samorajski, 1967). The axon diameter estimated from the axon

cross-sectional area was also related to the number of myelin lamellae for comparison with the above mentioned relationship

The aim of the present investigation was to examine whether undernutrition affects the axonal growth and the myelin deposition differently in pure motor and in pure sensory nerve tracts furthermore to study what effect nutritional rehabilitation will have upon these two fundamental structures of a nerve fibre.

II MATERIAL AND METHODS

Animals

Virgin rats of the Sprague Dawley strain 3 months old were mated with male rats of various ages. The time of fertilization was determined by vaginal smears twice daily. All litters from which rats were examined were adjusted to 8 pups at the time of birth. Only male rats were examined in the present study. Control and undernourished rats were examined at the age of 90 and 180 days. Prenatally undernourished rats nutritionally rehabilitated from birth to the age of examination were sacrificed at 90 and 180 days of age. Altogether 6 animals were investigated. The spinal roots were examined from 90-day-old animals and the optic nerve from 180-day-old rats.

Animal environment

All control rats and rats of various experimental groups were kept in plastic cages at a temperature of 25°C and in a relative humidity of 60 per cent. The room in which the rats were kept was illuminated from 6 a.m. to 6 p.m.

Diet

All rats were given pellets with a protein content of 18,8 cal per cent concentrated fish protein (949A Astra-Ewos, Södertälje, Sweden) and water ad libitum. The content of vitamins and salt constituents was above the minimum requirement. This was also true for the reduced amount of pellets given to rats subjected to undernutrition (cf Sjöma, 1974).

Undernutrition

The technique of undernutrition was that described by Chow and Lee (1964). This technique implies pre- and postnatal undernutrition through a maternal diet restriction amounting to 50 per cent of the amount consumed by a control mother. The nutritional restriction was initiated on the first day of gestation and lasted during pregnancy and lactation period. After weaning, the 8 pups were also subjected to a nutriment restriction of 50 per cent of the amount consumed by age-matched control rats. Undernutrition was extended to the age of investigation. All undernourished rats were allowed water *ad libitum*.

Nutritional rehabilitation

Prenatally undernourished litters were adjusted to 8 pups at the time of birth. From the first day post partum the mothers of these litters were allowed pellets *ad libitum* until weaning. After weaning the 8 pups had free access to pellets until they were sacrificed at 90 and 180 days of age. Rehabilitated rats were allowed water *ad libitum*.

Histological technique

The animals were anaesthetized with ether. They were perfused with a mixed solution of 1.0 per cent paraformaldehyde and 1.25 per cent glutaraldehyde in 0.15 M cacodylate buffer of pH 7.15 with added CaCl_2 . The amount used was 1.5–2.0 ml per 1 gram body weight. This was followed by perfusion with the same volume of solution four times the above mentioned concentrations. The nerves were then left in situ in the perfused animals for 4 hours, before they were dissected out. Thereafter the nerves were fixed in concentrated solution for another 12 hours and postfixed in 1.0 per cent osmium tetroxide for 2 hours, dehydrated and embedded in Epon 812 (cf Karnovsky 1965). One micron thick complete cross sections were cut with a LKB-ultratome from the middle level of the spinal roots and from the optic nerve 3 millimeter behind the eye-ball for light microscopic orientation. Immediately adjoining sections were examined in a Philips EM 200 electron microscope.

Counting techniques and presentation of the material

Electron micrographs were taken from a sector varying between 40 and 45 degrees of a cross-section of the spinal roots and of the optic nerves. Random sampling was applied to every second (spinal roots) and every third (optic nerves) grid mesh across the entire sectors. Total magnification of prints used for examination varied from 26 000 to 120 000 times. The magnification was controlled with the aid of a calibration suspension containing 0.5 micron large latex spheres (Ernest F. Fullam Inc. Schenectady N.Y. U.S.A.) which was placed on the grid before examination. Only micrographs in which all lamellae of the myelin sheath were clearly visible were analysed. The number of myelin lamellae was counted from the innermost to the outermost dense line. The axon circumference measured with a map meter was only analysed in fibres cut perpendicular to their long axis as judged by circular appearances of microtubules (figure 1 and ?). The number of axons and appurtenant number of myelin lamellae examined ranged per each nerve from 63 to 168. Lamellar counts per each nerve ranged from 168 to 437 myelin sheaths. Axon circumference was plotted for each nerve against the number of myelin lamellae (figures 3 to 5). Number of myelin lamellae indicating myelin sheath thickness, was illustrated in frequency distribution diagrams in intervals of 5 or 10 lamellae and expressed as percentages of the number of total myelin sheaths counted (figures 6 to 8). From axons where the circumference was determined the

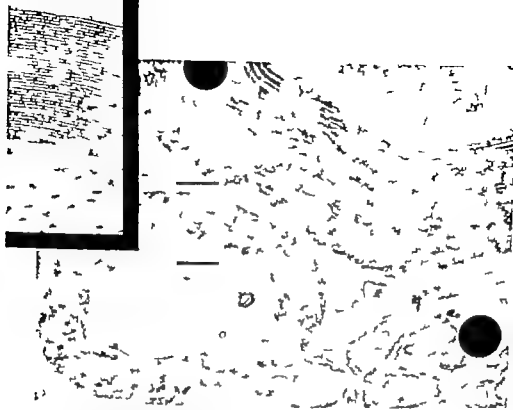


Fig 1 Myelinated axon from the ventral root of 90-day-old control rat. Upper left, higher magnification of the framed part of the myelin sheath. Black spheres were used for magnification control (20,000X and 62,000X)

cross-sectional area was also calculated with the aid of a planimeter. From the value of the axon area the diameter was determined according to the formula

$$d = \sqrt{\frac{4A}{\pi}}$$

The complete material of estimated axon circumferences, axon areas and diameters with appurtenant lamellar counts is presented in table I to IX. The additional lamellar counts are given in table X to XII.

Statistical methods

The Fisher permutation test for independent two-sample case with Edgeworth's expansion was used in order to assess the difference between control and experimental rats with respect to axon circumferences and axon diameters at given numbers of myelin lamellae. The X values corresponding to myelin sheath thickness



Fig. 2 Myelinated axons from the optic nerve of an undersized 180-day-old rat. Black sphere was used for control of magnification (86,000X)

were subgrouped in groups of almost equal magnitudes. The comparisons within the subgroups were then pooled together. This statistical analysis was chosen in order to avoid the following assumptions of traditional analysis: (a) normal distribution, (b) linear regression and (c) homogeneous variances. For statistical evaluation of the frequency distributions of myelin lamellae, test for trend in a contingency table with p-value determination by Edgeworth's expansion and correction for continuity (Odén and Wedel 1973) was used. Statistical evaluation was performed with the aid of an IBM 360 computer at Gothenburg University Computing Centre.

III RESULTS

Ventral root

The relationship between axon circumference and myelin sheath thickness expressed as the number of myelin lamellae showed a nonlinear relationship in the ventral root. This was true for both control undernourished and rehabilitated rats. In all three nerves an S-shaped relationship could be noted. Among thin fibres with the same axon circumference the myelin sheath thickness varied within relatively wide limits. In thick fibres the axon circumference varied more than did the myelin sheath thickness (figure 3). This nonlinear relationship was more pronounced when the axon diameter calculated from the axon cross-sectional area was related to the myelin sheath thickness. In normal and rehabilitated rats no fibres were observed in the ventral root containing more than 140 lamellae. In the undernourished rat the thickest myelin sheaths of the ventral root contained 130 lamellae. Undernourished and rehabilitated rats showed significantly ($p < 0.05$) thinner myelin sheaths in proportion to axon circumference when compared to the control rat. This was also true when myelin sheath thickness was related to axon diameter. No significant difference could be obtained between undernourished and rehabilitated rats in the relation of myelin sheath to axon circumference or to axon diameter (figure 3d).

In a representative part of all myelinated fibres of the ventral root the myelin sheath thickness varied between 11 and 140 lamellae in the control rat. Two distinct peaks could be noted in the frequency distribution diagram (figure 6) one for myelin sheaths 41–50 lamellae thick and one for sheaths containing 121–130 lamellae.

In the undernourished rat the myelinated fibres were surrounded by myelin sheaths varying from 11 to 130 lamellae thick. Even in the undernourished rat two peaks could be distinguished, one for sheaths consisting of 51–60 lamellae and one for sheaths containing 111–120 lamellae. The myelin sheath thickness of the undernourished rat was significantly thinner ($p < 0.001$) than in the control rat. In the undernourished and subsequently rehabilitated rat no fibres were observed which contained less than 31 lamellae. The thickest myelin sheaths consisted of 140 lamellae. The two peaks in the frequency distribution diagram (figure 6) were contributed by myelin sheaths containing 51–60 and 91–100 lamellae. Thus the rehabilitated rat did not show a full recovery of the myelin sheaths when compared to the control rat. However no significant difference could be obtained between myelin sheath thicknesses of rehabilitated and control rats. ($p > 0.2$)

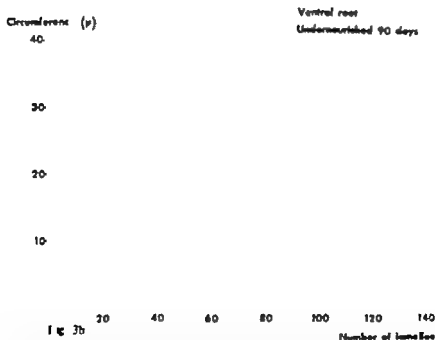
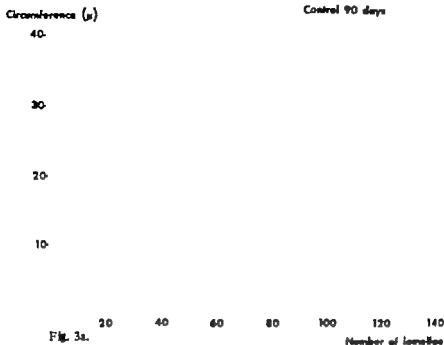


Fig. 3 Relationship between axon circumference and myelin sheath thickness, expressed as number of myelin lamellae in ventral roots. (a) Control rat (b) undernourished rat, (c) rehabilitated rat, (d) comparison between respective regression lines. These regressions were calculated without taking into account the assumptions of traditional analysis (see text). Undernourished and rehabilitated rat show significantly ($p < 0.05$) thinner myelin sheaths in proportion to axon circumferences in comparison to the control rat. No significant difference in this proportion can be obtained between undernourished and rehabilitated rats.

Circumference (μ)

40

30

20

10

Ventral root

Rehabilitated 0-90 days

Fig. 3c.

20

40

60

80

100

120

140

Number of lamellae

Circumference (μ)

40

30

20

10

Ventral root — Control

90 days

— Undernourished

90 days

— Rehabilitated

0-90 days

Fig. 3d.

20

40

60

80

100

120

140

Number of lamellae

Dorsal root

The relationship between axon circumference and number of myelin lamellae was also slightly S-shaped in the dorsal root. This nonlinear relationship was also valid for the dorsal root fibres of experimental rats (figure 4). When comparing the axon diameter with the number of myelin lamellae no strict linear relationship was noted in control or undernourished rats, nor in the rehabilitated rat. In undernourished and rehabilitated rats the proportion between axon circumference or axon diameter and myelin sheath thickness was not altered when compared with the control rat ($0.1 < p < 0.6$). See figure 4d.

The myelin sheath thickness expressed as the number of myelin lamellae ranged between 10 and 130 lamellae in all three nerves. Two peaks could be distinguished in the myelin sheath frequency distribution (figure 7) both in control and in experimental rats. In the control rat these appeared for sheaths containing 41–50 and 101–110 lamellae. In the undernourished rat for sheaths consisting of 31–40 and 81–90 lamellae. The rehabilitated rat exhibited the peaks for sheaths consisting of 31–40 lamellae and for those made up by 91–100 lamellae. Accordingly the deposition of myelin in the undernourished rat was markedly retarded. In the rehabilitated rat no significant recovery could be observed as far as myelin deposition was concerned. When compared to the control rat the myelin sheath thickness of the dorsal root fibres of the rehabilitated rat was significantly thinner ($p < 0.001$).

Optic nerve

In this central nerve tract the relationship between axon circumference and the number of myelin lamellae was almost linear in control and rehabilitated rats. In the undernourished rat the axons surrounded by the same number of myelin lamellae varied within a wide range concerning their circumferences and the linear relationship was less pronounced (figure 5). The axon diameter calculated from the axon area, was related in the same way to the myelin sheath thickness in control and rehabilitated rats as was the circumference. In the undernourished rat however the linear relationship was distorted and the nerve fibres with the same number of myelin lamellae showed great variations in axon diameters. Comparing the optic nerves of control and of undernourished rats (figure 5d) the fibres of the undernourished rat showed disproportionately thicker myelin sheaths when compared with the fibres of the control rat ($p < 0.01$). Only in the largest fibres containing 30 myelin lamellae or more the myelin sheath thickness of the undernourished rat showed almost normal proportions towards the axon circumference and axon diameter. In the rehabilitated rat the myelin sheath thickness was disproportionately thicker in both thin and thick fibres ($p < 0.01$) (figure 5d).

The lamellar counts of the optic nerves showed a unimodal distribution in both control and experimental rats (figure 8). In all three nerves the peak in the frequency distributions was made up by sheaths consisting of 6 to 10 lamellae. In control and rehabilitated rats the myelin sheath thickness ranged between 1 and 46

lamellae. In the undernourished rat the thickest sheaths consisted of 35 lamellae. The myelin deposition was significantly impaired in the undernourished rat ($p < 0.001$) as compared with the control rat, whereas in the rehabilitated rat this impairment in myelin deposition was completely recovered.

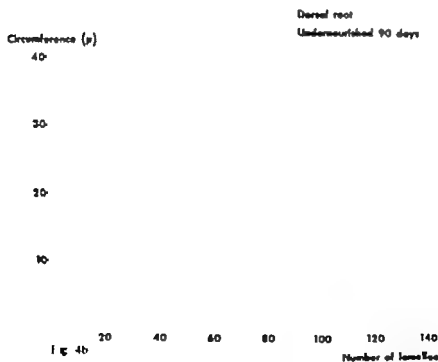
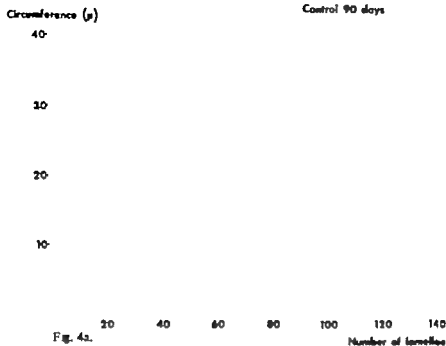
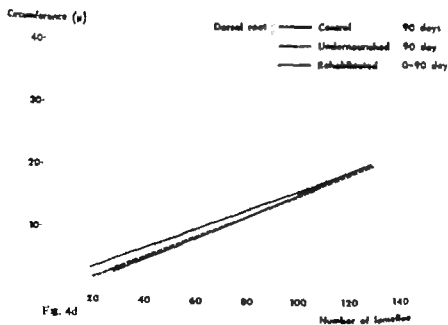
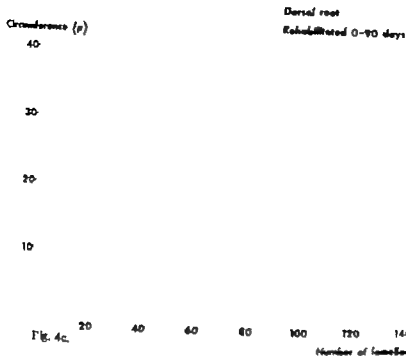


Fig. 4 Relationship between axon circumference and myelin sheath thickness, expressed as number of myelin lamellae in dorsal roots (a) Control rat (b) undernourished rat (c) rehabilitated rat, (d) comparison between respective regression lines. These regressions were calculated without taking into account the assumptions of traditional analysis (see text). No significant difference can be obtained between the proportion axon circumference to myelin sheath thickness in any of the nerves.



Circumference (μ)

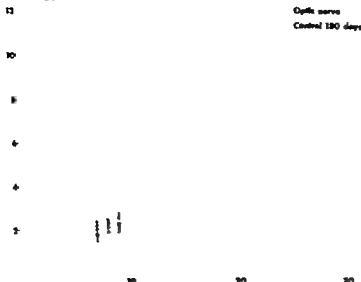


Fig. 5a.

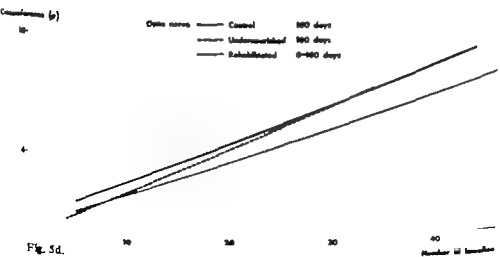
Circumference (μ)



Fig. 5b

Number of lamellae

Fig. 5 Relationship between axon circumference and myelin sheath thickness in optic nerves (a) Control rat, (b) undernourished rat (c) rehabilitated rat (d) comparison between respective regression lines. These regressions were calculated without taking into account the assumptions of traditional analysis (see text). Undernourished and rehabilitated rats show significantly ($p < 0.01$) lesser axon circumferences in proportion to myelin sheath thicknesses, in comparison to the control rat. No significant difference in this proportion can be obtained between undernourished and rehabilitated rats.



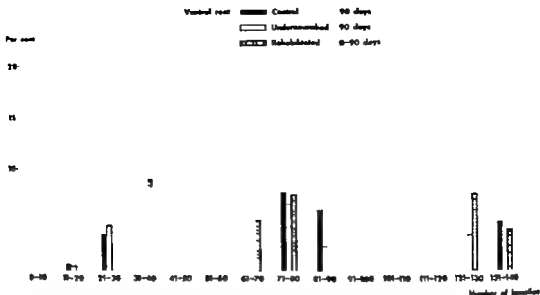


Fig. 6 Frequency distribution of pooled myelin sheath thicknesses in ventral roots. The myelin sheath thicknesses are significantly thinner ($p < 0.001$) in the undernourished rat as compared to the control rat. No significant difference could be obtained between rehabilitated and control rats. Note the absence of thin myelin sheaths of the rehabilitated rat.

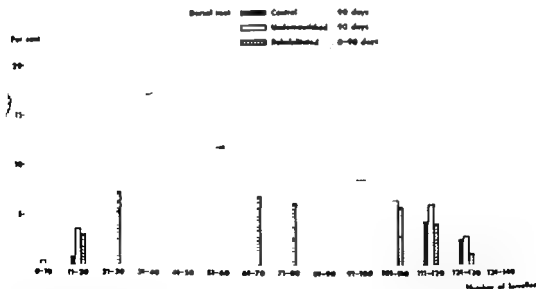


Fig. 7 Frequency distribution of pooled myelin sheath thicknesses in dorsal roots. The myelin sheath thicknesses of undernourished and rehabilitated rats are significantly thinner ($p < 0.001$) than those of the control rats.

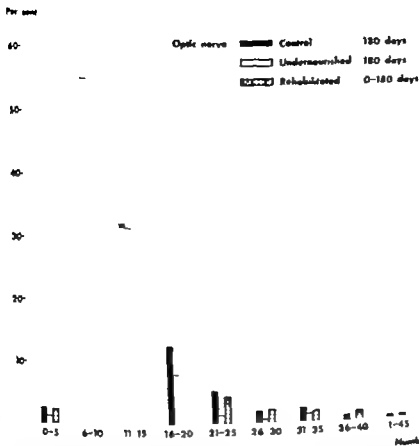


Fig. 8. Frequency distribution of pooled myelin sheath thickness in optic nerves. In the undernourished rat the myelin sheath thicknesses are significantly thinner ($p < 0.001$) than in the control rat. No difference can be noted between myelin thicknesses in rehabilitated and control rats.

IV DISCUSSION

Pre and postnatal undernutrition showed to have retarding effects upon the deposition of myelin in ventral and dorsal spinal roots and in the optic nerve. In the ventral root the impairment in myelin deposition was almost entirely restored after nutritional rehabilitation but in the dorsal root no significant recovery could be detected after rehabilitation. In the optic nerve this retardation in myelination was fully recovered by nutritional rehabilitation initiated at birth. Hedley Whyte (1973) demonstrated that severe food deprivation during the suckling period affects the myelination in the rat sciatic nerve adversely and that this deficit in myelin deposition showed to be partially reversible during subsequent nutritional rehabilitation. When it is taken into account that the ventral and dorsal roots of the fifth lumbar segment merge into the sciatic nerve the results concerning the spinal roots are in agreement with those of Hedley Whyte. Hence the findings in the sciatic nerve by Hedley Whyte may represent a summation of the effects of undernutrition and subsequent rehabilitation on myelin deposition of the ventral and dorsal roots.

The deficit in myelination found in the sciatic nerve must accordingly have been initiated during the suckling period. In the present study the rats were subjected to a prenatal undernutrition and rehabilitated from birth through food ad libitum to the lactating mother. Therefore the irreversible deficit found in the deposition of myelin in the dorsal root may have been initiated before birth. During both late fetal life and early suckling period Schwann cell proliferation and subsequent commencement of myelination is known to occur (Asbury 1967, Friede and Samorajski 1968). Therefore nutritional deprivation initiated during fetal life or during early suckling period may affect Schwann cell proliferation and early myelination, since it is known that periods of active cell proliferation is most vulnerable towards undernutrition (Dobbing 1968).

In the histogram of myelin sheath distribution of the ventral root it is apparent that the rehabilitated rat lacks thinly myelinated fibres when compared to control and undernourished rats. This phenomenon was also observed to a lesser extent in the sciatic nerve fibres of rehabilitated rats by Hedley Whyte (1973). A plausible explanation to this phenomenon given by Hedley Whyte is that undernutrition may decrease the rate of mitosis of Schwann cells and when rehabilitation is initiated Schwann cells with myelinating axons proceed with myelination but no new axons will be myelinated. In the dorsal root thinly myelinated fibres were observed also in the rehabilitated rat. This difference between ventral and dorsal spinal roots of rehabilitated rats is not known. A possible explanation may be that the period of Schwann cell multiplication in the dorsal root is more prolonged than in the ventral

root, since it is known that the fibre calibre growth proceeds at a slower rate in the dorsal than in the ventral root (Sima 1974). The rate of mitotic activity of Schwann cells may therefore not be so severely affected by undernutrition as assumed for the ventral root.

A linear relationship between axonal circumference and myelin sheath thickness in normal developing rat sciatic has been demonstrated by Clos and Legrand (1970), Friede and Samorajski (1968) and by Hedley Whyte (1973). However Webster (1971) and Webster and O'Connell (1970) found that the axon diameter was not proportional to the myelin sheath thickness. In the present study no linear relationship was found between axon circumference or axon diameter estimated from the axon cross-sectional area and the myelin sheath thickness in the ventral or in the dorsal root. Differences may thus exist in the mode of myelin deposition in the sciatic nerve on one hand and in the ventral and the dorsal roots on the other hand. It may therefore be assumed that axonal growth is not the only factor controlling the myelin deposition at least in the ventral and dorsal roots as proposed by Friede (1972) for the sciatic nerve.

In the ventral root the deposition of myelin was more affected by early instituted protein-calorie undernutrition than the axonal expansion. Even in the rehabilitated rat the myelin sheaths were thinner in proportion to the axon circumference when compared to the control rat which may indicate an early reduced myelinating potential of the Schwann cells of the ventral root. This is in agreement with the findings by Hedley Whyte (1973) which demonstrated that the myelin sheath growth of sciatic nerve fibres was retarded to a great extent than the axonal growth in undernourished rats. In the dorsal root, however, the myelin sheath and the axon circumference were affected proportionately in both undernourished and rehabilitated rats when compared to the control rat.

In the optic nerve fibres the myelin deposition was significantly retarded by undernutrition. This retardation was completely recovered by nutritional rehabilitation initiated at the time of birth. Such a restoration of the myelin sheath was also expected, since it is known that the oligodendrocytes initiating the myelination first appear just prior to the time when myelination begins at day 7 to 9 postnatally (Vaughn, 1969) and therefore the differentiation of oligodendrocytes would not be affected by undernutrition during the prenatal period. Between axon circumference and myelin sheath thickness there existed an almost linear relationship in the optic nerves of control and rehabilitated rats. In the undernourished rat this relationship was distorted with a wide spread in the proportion between axon circumference and myelin sheath thickness in individual fibres. In both undernourished and rehabilitated rats the axonal expansion was more affected than was the myelin sheath. Thus in the optic nerve the converse relationship was valid concerning the effect of undernutrition upon axonal growth and myelin sheath when compared to the ventral motor root in which the myelination was more affected than the axonal growth by undernutrition.

When comparing the relationship between axon circumference and myelin sheath thickness on one hand and that between axon diameter and myelin sheath

thickness on the other hand no marked differences were at hand in any of the three nerves investigated indicating that the perpendicularly cut axon may be regarded as roughly circular

The results of the present study fit well with earlier investigations on total fibre diameters in undernourished and rehabilitated rats (Sima, 1974 Sima and Sourander 1974) In these studies it was found that the calibre growth of the dorsal root and the optic nerve were irreversibly retarded by undernutrition whereas the impairment of the ventral root fibre growth was fully restored by nutritional rehabilitation. Only in the optic nerve does the complete restoration of the myelin deposition contrasts with the irreversibly retarded total calibre growth This difference may be explained by the fact that the thickness of the myelin sheath of an optic nerve fibre only constitutes a very small percentage of the total fibre diameter Therefore the difference in the myelin sheath thickness between undernourished and rehabilitated rats may not have been detected by the method used in the earlier study (Sima and Sourander 1974)

The cause of the different effect of nutritional rehabilitation on myelination in the ventral and dorsal roots of previously undernourished rats remains obscure One may speculate on differences in the mode of Schwann cell proliferation and early myelination and assume that these processes are affected differently by undernutrition in the ventral and the dorsal roots. This difference in susceptibility may perhaps be mediated by factors like different timing of Schwann cell proliferation in relation to birth or by differences in enzymatic patterns or in hormonal response of the Schwann cells. It is known that hypothyroidism may cause retardations in cell proliferation for instance in the cerebellum (cf Hayrs and Taylor 1951 Legrand, 1967) This latter mechanism has however been denied as a cause of retardation in Schwann cell proliferation and myelination (Clos and Legrand, 1970) Hypothyroidism is not conceivable as being the underlying cause in the present study since the thyroid essentially develops just after birth in the rat (cf Miller 1969). However other regulatory mechanisms, like for instance the role played by insulin which can be secreted already during the midgestation period in the fetal rat must be considered when pondering on the cause of this topographic difference in myelination of undernourished rats.

V SUMMARY

The effect of pre and postnatal undernutrition on myelin deposition and axonal radial growth was examined ultrastructurally in the motor ventral root and the sensory dorsal root of the fifth lumbar segment in adult rats. In addition the same effect was studied on the corresponding structures of adult rat optic nerve fibres. In order to evaluate whether achieved deficits were recoverable nutritional rehabilitation was initiated at birth. From the results it is concluded that

1. Pre and postnatal undernutrition causes impairments in myelin deposition in ventral and dorsal root fibres of the fifth lumbar segment (LV) and in the nerve fibres of the optic nerve
2. The deficit in myelination is completely recovered in the optic nerve fibres and almost entirely restituted in the ventral root fibres of the fifth lumbar segment (LV). In the nerve fibres of the dorsal spinal root of the same segment the deposition of myelin is irreversibly distorted by early undernutrition
3. In the ventral root nerve fibres (LV) the myelin deposition is proportionately more affected by undernutrition than the axonal expansion. The relation axonal radial growth to myelin deposition is affected proportionately by undernutrition concerning the nerve fibres of the dorsal spinal root (LV). In the nerve fibres of the optic nerve the axonal radial growth is proportionately more affected by pre and postnatal undernutrition than the deposition of myelin

Furthermore it is suggested that differences may exist in the mode of Schwann cell proliferation in the ventral and dorsal spinal roots (LV) of the rat.

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VII TABLES

Table I Ventral root of control rat 90 days old

Number of Lamellae	Circumference μ	Area μ^2	Diameter μ	Number of Lamellae	Circumference μ	Area μ^2	Diameter μ
17	3.61	1.00	1.13	80	8.00	4.11	2.29
29	5.43	2.09	1.63	83	8.65	4.59	2.42
29	3.64	0.95	1.10	88	26.00	31.26	6.31
30	4.15	1.31	1.29	90	16.00	15.68	4.47
31	5.36	2.06	1.62	91	17.40	14.41	4.28
32	8.40	3.93	2.24	91	12.00	7.26	3.04
33	7.14	3.35	2.07	93	13.20	10.28	3.62
35	4.91	2.08	1.63	97	8.40	3.25	2.03
35	6.00	1.92	1.56	97	16.90	15.41	4.39
36	3.29	0.73	0.96	98	9.60	6.35	2.84
38	8.38	3.94	2.24	98	10.40	5.65	2.68
38	16.40	18.12	4.80	99	9.60	5.02	2.53
40	3.17	0.74	0.97	102	22.00	29.35	6.11
41	6.43	2.57	1.81	104	8.40	3.06	1.97
41	9.71	4.97	2.52	104	10.40	4.11	2.29
43	7.24	2.96	1.94	104	19.80	22.53	5.36
45	7.00	3.16	2.01	108	23.20	36.76	6.84
48	9.60	5.90	2.74	108	15.20	14.20	4.25
50	5.79	1.76	1.51	110	22.40	34.25	6.60
51	5.00	2.28	1.70	110	13.93	13.53	4.15
57	11.60	5.31	2.60	110	18.80	2.93	5.40
58	7.57	3.43	2.10	111	18.00	18.79	4.89
58	9.80	5.68	2.69	115	25.00	42.16	7.33
59	13.20	5.73	2.70	116	22.80	30.34	6.22
63	6.43	3.01	1.96	121	16.80	18.93	4.91
69	11.11	5.07	2.54	122	17.78	17.16	4.68
74	12.00	7.01	2.99	125	16.61	20.45	5.10
76	6.18	2.49	1.78	125	27.20	4.83	7.39
76	15.20	8.28	3.23	130	20.40	27.00	5.86
76	8.40	3.48	2.11	131	2.00	32.40	6.42
77	11.60	6.29	2.83	131	16.41	17.07	4.66
79	11.20	5.92	2.74	134	13.39	13.88	4.14

Table II Ventral root of undernourished rat 90 days old

Number of Lamellae	Circumference μ	Area μ^2	Diameter μ	Number of Lamellae	Circumference μ	Area μ^2	Diameter μ
18	3.38	0.82	1.01	38	4.60	2.70	1.85
2	6.63	2.37	1.75	44	7.00	2.33	1.71
23	4.00	1.00	1.13	44	7.67	4.62	2.42
25	14.00	5.03	2.52	45	6.80	2.00	1.60
8	5.60	1.40	1.34	51	5.00	2.04	1.60
30	4.80	1.60	1.43	53	5.60	1.90	1.56
34	6.00	2.93	1.92	53	21.20	1.81	5.27
35	9.40	2.57	1.82	54	6.80	2.90	1.92
35	4.67	1.72	1.47	60	6.20	1.60	1.43
35	6.67	1.76	1.51	60	7.60	4.00	2.66
36	1.60	1.95	2.26	62	6.67	3.13	1.99
36	5.00	1.89	1.56	83	18.40	8.00	3.33
36	4.75	1.26	1.29	65	8.00	3.90	2.33
37	1.33	1.1	1.64	65	6.40	2.43	1.75

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
70	10.80	6.80					
77	10.00	5.40	94	114	13.67	15.82	4.49
82	7.60	3.30	2.6	114	28.40	48.89	7.89
83	19.17	17.61	2.05	115	21.33	37.96	6.96
91	15.20	13.30	4.74	115	29.60	58.90	8.66
95	20.40	26.80	4.12	117	25.60	36.63	6.83
99	20.00	24.59	5.84	118	20.80	30.20	6.20
100	16.80	18.80	5.60	118	18.17	26.4	5.78
101	17.00	21.17	4.89	118	22.00	32.00	6.38
102	20.00	26.43	5.20	118	19.50	8.02	5.97
107	19.00	30.87	5.80	118	21.00	35.78	6.75
110	19.00	27.08	6.27	119	26.80	39.47	7.09
110	18.67	28.89	5.88	119	20.83	9.73	6.15
110	18.67	28.19	6.07	121	22.00	34.80	6.66
112	22.80	30.80	5.99	121	23.20	36.70	6.84
112	28.40	50.55	6.46	123	29.20	56.70	8.50
113	23.60	35.60	8.03	126	25.40	41.20	7.24
			6.73				

Table III. Ventral root of rehabilitated rat, 90 days old.

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
31	3.20	0.60	0.87	62	18.00	24.60	5.60
32	7.60	3.90	2.23	63	13.40	8.10	3.21
33	3.70	1.10	1.18	64	7.20	3.30	2.05
34	7.60	3.10	1.99	65	10.40	6.70	2.92
36	7.40	2.80	1.89	66	9.70	7.00	2.99
36	10.00	4.00	2.26	69	13.20	6.10	2.79
38	6.30	1.50	1.38	71	8.30	5.50	2.65
40	12.00	3.00	1.95	72	14.00	8.90	3.37
40	8.00	4.38	2.36	72	11.60	7.90	3.17
40	9.40	5.90	2.74	78	13.60	6.20	2.81
41	6.50	2.60	1.82	79	24.80	33.80	6.56
41	6.33	3.30	2.05	80	10.30	6.10	7.9
43	7.00	3.40	2.08	85	9.60	7.10	3.01
43	10.20	5.00	2.52	87	9.50	8.70	3.31
46	8.00	4.60	2.42	87	15.20	8.30	3.5
46	7.20	3.60	2.14	90	17.80	19.00	4.92
48	11.20	6.70	2.92	91	13.80	13.20	4.10
48	7.60	4.10	2.29	92	21.60	27.60	5.93
48	8.80	4.10	2.29	93	11.67	11.60	3.84
52	12.70	6.60	2.90	94	17.30	23.80	5.50
52	11.20	5.60	2.67	95	20.40	27.80	5.95
55	6.80	3.00	1.95	100	25.80	38.10	6.97
57	9.20	5.00	2.52	101	22.60	33.20	6.40
58	7.00	4.10	2.29	101	26.80	44.70	7.55
58	9.00	5.80	2.72	102	22.00	33.90	6.57
59	9.20	4.40	2.37	103	17.00	24.70	5.61
60	12.00	8.50	3.29	103	20.80	30.70	6.25
60	11.70	7.60	3.11		23.80	36.80	

Number of Lamellae	Circumference μ	Area μ^2	Diameter μ	Number of Lamellae	Circumference μ	Area μ^2	Diameter μ
103	25.80	42.50	7.36	114	26.40	46.80	7.72
104	25.20	39.60	7.10	114	22.00	33.00	6.48
107	21.00	27.50	5.92	116	22.80	32.60	6.44
111	25.20	43.80	7.47	116	21.00	36.70	6.84
111	12.30	11.60	3.84	118	22.80	36.10	6.78
111	21.20	29.00	6.08	119	20.40	28.80	6.06
112	22.80	33.40	6.52	119	27.60	52.50	8.18
11	15.20	14.80	4.34	125	22.80	33.10	6.49
114	31.20	63.20	8.97				

Table IV Dorsal root of control rat, 90 days old

Number of Lamellae	Circumference μ	Area μ^2	Diameter μ	Number of Lamellae	Circumference μ	Area μ^2	Diameter μ
28	4.00	1.36	1.34	88	12.60	7.30	3.05
34	12.00	11.65	3.86	89	15.60	16.60	4.60
34	4.00	1.50	1.38	94	8.80	5.59	2.67
35	4.00	1.44	1.34	95	10.67	7.40	3.07
38	7.60	3.27	.05	97	10.67	9.01	3.39
39	5.60	1.92	1.56	97	12.20	8.30	3.25
39	4.10	1.33	1.29	99	9.00	5.30	2.60
40	7.20	3.40	2.08	99	16.20	15.10	4.39
41	5.20	1.56	1.43	99	18.00	22.90	5.40
42	4.67	1.89	1.56	99	12.80	10.50	3.66
43	6.60	2.00	1.60	100	11.00	10.04	3.57
43	5.80	2.30	1.71	100	20.00	31.51	6.33
44	5.60	1.94	1.56	101	12.20	9.91	3.55
45	6.00	1.97	1.60	101	12.83	12.20	3.94
45	6.60	2.10	1.64	102	14.60	15.30	4.41
47	6.00	2.68	1.85	103	21.33	26.79	5.84
48	8.40	2.59	1.82	103	29.80	20.50	5.11
49	5.33	1.34	1.29	103	22.00	29.78	6.16
49	6.00	1.47	1.38	104	26.57	42.04	7.31
49	9.00	3.60	2.14	106	13.67	14.86	4.36
49	4.40	1.29	1.29	106	16.40	14.80	4.34
5	6.40	2.34	1.71	106	14.60	12.80	4.04
53	6.00	2.50	1.78	106	15.00	15.60	4.46
53	11.33	5.34	2.60	107	16.00	19.77	5.02
55	6.80	.75	1.89	109	17.00	17.74	4.75
59	8.80	4.70	2.45	110	24.40	36.21	6.79
61	10.80	6.10	2.79	111	2.60	27.00	5.86
67	7.80	3.77	2.20	112	17.40	18.91	4.91
70	6.67	3.69	.17	114	19.00	29.21	6.10
80	10.83	10.22	3.60	120	15.80	16.40	4.57
82	8.80	5.19	.57	121	24.00	39.67	7.11
83	10.57	6.2	.83	13	22.33	44.52	7.53
86	1.40	8.90	3.37	13	24.00	34.90	6.67
86	11.60	9.50	3.48	124	3.80	38.91	7.04
88	11.33	10.5	3.64	127	17.20	0.58	5.1
88	9.80	6.50	2.88				

Table V Dorsal root of undernourished rat, 90 days old

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
18	3.00	0.55	0.87	92	13.40	11.17	3.78
19	3.50	1.03	1.13	92	13.40	11.09	3.76
20	2.60	0.55	0.87	93	9.80	5.77	2.72
25	3.60	0.96	1.13	94	11.80	8.58	3.31
32	4.83	1.73	1.47	94	16.60	18.91	4.91
32	4.00	0.55	0.87	94	17.75	14.84	4.34
33	4.60	1.11	1.18	97	11.40	8.11	3.21
37	5.00	1.95	1.60	98	13.20	11.76	3.88
38	4.80	1.70	1.47	98	12.80	11.32	3.79
38	6.00	2.43	1.75	98	15.40	16.57	4.60
41	10.00	2.22	1.67	98	15.40	16.57	4.60
47	6.20	1.75	1.51	99	11.60	9.44	3.46
51	13.80	10.30	3.62	103	17.20	18.38	4.84
57	8.60	4.94	2.50	103	16.60	17.97	4.79
67	11.20	6.67	2.92	103	17.40	22.40	5.34
68	11.40	6.86	2.96	104	21.20	25.44	5.69
69	9.00	4.66	2.45	107	9.60	5.50	2.65
69	9.40	4.77	2.47	107	12.80	9.54	3.48
73	10.80	6.64	2.90	109	21.20	29.23	6.10
73	11.80	8.64	3.31	111	21.80	20.53	5.11
76	9.60	6.48	2.88	112	24.60	33.83	6.56
76	11.00	7.81	3.15	112	14.80	14.62	4.31
78	9.00	3.96	2.26	113	18.80	24.82	5.62
79	9.80	6.14	2.79	115	15.50	21.29	5.21
80	10.67	8.75	3.34	115	23.20	29.05	6.09
82	10.20	6.01	2.76	117	16.17	22.90	5.40
83	11.00	7.87	3.17	117	18.80	21.09	5.18
84	11.00	7.83	3.16	117	22.60	35.50	6.72
86	12.40	10.21	3.60	118	20.60	20.38	5.10
86	13.40	12.54	3.99	119	22.00	29.12	6.09
86	14.60	11.91	3.89	121	17.00	19.94	5.03
88	15.40	15.47	4.44	123	21.40	30.18	6.20
88	11.00	8.42	3.27	124	27.80	50.28	8.00
88	8.40	5.33	2.60	124	17.60	16.46	4.58
90	16.40	17.65	4.75	124	20.60	26.30	5.79
90	11.40	8.51	3.29	128	22.20	32.54	6.43

Table VI Dorsal root of rehabilitated rat, 90 days old

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
19	3.70	1.40	1.34	41	8.40	2.00	1.60
30	3.30	0.90	1.07	41	7.20	3.10	1.99
32	4.40	2.60	1.82	41	5.00	1.40	1.34
38	9.60	2.70	1.85	41	8.40	3.70	2.17
40	4.00	1.30	1.29	44	11.00	3.00	1.95
40	6.30	1.60	1.43	49	11.60	7.90	3.17
40	5.60	1.70	1.47	50	9.50	6.10	2.79
40	5.20	1.80	1.51	54	11.20	6.00	2.76

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
57	9.20	3.10	1.99	100	17.20	19.60	5.00
67	1.40	6.90	2.96	100	16.00	15.50	4.44
71	11.20	5.80	2.72	101	12.40	9.40	3.46
7	10.00	7.30	3.05	101	20.40	20.60	5.12
73	11.00	9.30	3.44	103	17.20	18.70	4.88
76	11.20	7.50	3.09	103	11.30	10.90	3.73
77	12.80	8.10	3.21	104	16.30	20.20	5.07
8	14.00	14.40	4.28	105	14.00	15.10	4.39
82	12.00	8.10	3.11	107	16.00	15.10	4.39
82	16.80	18.30	4.83	107	19.20	21.10	5.18
83	11.20	7.20	3.03	108	18.40	22.40	5.34
84	14.40	12.10	3.93	108	16.80	19.20	4.95
87	27.0	14.60	4.31	109	19.60	25.50	5.70
90	14.40	13.70	4.18	110	21.40	28.80	6.06
91	11.60	8.30	3.25	111	22.40	30.00	6.18
9	1.40	9.70	3.5	111	13.70	14.60	4.31
92	14.40	13.50	4.15	112	22.00	31.70	6.35
93	13.60	10.70	3.69	112	18.80	22.60	5.37
93	19.40	21.50	5.23	112	16.80	24.00	5.53
93	20.00	20.50	5.11	112	21.00	35.20	6.68
96	12.20	12.10	3.93	114	16.40	17.00	4.65
96	13.00	13.50	4.15	116	22.00	27.60	5.93
97	15.80	16.70	4.61	121	19.60	23.60	5.48
98	14.40	11.80	3.88	123	18.30	28.20	6.00
99	11.70	9.50	3.48	123	22.60	31.70	6.35
99	13.20	13.30	4.12				

Table VII Optic. nerve of control rat, 180 days old

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
5	1.73	0.1	0.52	7	2.36	0.43	0.74
5	2.00	0.32	0.64	7	2.82	0.63	0.90
5	1.81	0.20	0.50	7	2.05	0.30	0.62
5	1.62	0.1	0.52	7	1.68	0.21	0.52
5	1.91	0.24	0.55	7	2.18	0.28	0.60
5	2.05	0.24	0.55	7	3.00	0.67	0.92
6	1.27	0.08	0.32	7	2.00	0.21	0.52
6	3.45	0.71	0.95	7	1.71	0.15	0.44
6	1.77	0.24	0.55	7	1.81	0.25	0.56
6	2.18	0.35	0.67	7	1.81	0.25	0.56
6	1.55	0.19	0.49	7	2.38	0.48	0.78
6	2.38	0.34	0.66	7	1.76	0.22	0.53
6	2.24	0.29	0.61	7	2.29	0.35	0.67
6	2.14	0.33	0.65	7	1.62	0.25	0.56
6	1.81	0.18	0.48	7	1.86	0.21	0.52
6	1.91	0.25	0.56	7	2.43	0.38	0.70
7	1.46	0.14	0.42	7	2.00	0.28	0.60
7	3	0.25	0.56	7	1.76	0.19	0.49
7	2.00	0.28	0.60	8	2.73	0.46	0.77
7	1.81	0.24	0.55	8	2.46	0.34	0.66

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
8	1.23	0.11	0.37	10	1.43	0.14	0.42
8	3.09	0.71	0.95	10	2.48	0.39	0.70
8	2.36	0.41	0.72	10	2.86	0.53	0.82
8	2.18	0.28	0.61	10	2.67	0.39	0.66
8	2.54	0.47	0.77	10	2.19	0.36	0.68
8	1.91	0.30	0.62	10	2.10	0.24	0.55
8	2.00	0.27	0.59	10	2.24	0.33	0.65
8	1.64	0.21	0.52	11	1.82	0.24	0.55
8	1.91	0.29	0.61	11	2.73	0.60	0.87
8	2.32	0.34	0.66	11	2.00	0.24	0.55
8	2.32	0.29	0.61	11	2.72	0.43	0.74
8	2.36	0.30	0.62	11	3.00	0.62	0.89
8	2.05	0.24	0.55	11	3.48	0.65	0.91
8	2.38	0.36	0.68	11	1.91	0.29	0.61
8	2.67	0.44	0.75	12	2.09	0.31	0.63
8	2.71	0.49	0.79	12	2.27	0.31	0.63
8	2.10	0.23	0.54	12	2.50	0.46	0.77
8	2.29	0.17	0.47	12	3.18	0.59	0.87
8	3.48	0.55	0.84	12	3.72	0.78	1.00
8	2.24	0.36	0.68	12	3.14	0.57	0.85
8	2.29	0.33	0.65	12	7.38	0.86	1.05
8	2.33	0.33	0.65	12	2.76	0.50	0.80
8	1.81	0.23	0.54	13	3.73	0.95	1.10
8	2.05	0.35	0.67	13	2.10	0.26	0.58
9	2.00	0.35	0.67	13	2.57	0.51	0.81
9	2.18	0.33	0.65	14	2.68	0.54	0.83
9	3.18	0.51	0.76	14	3.23	0.73	0.96
9	2.27	0.33	0.65	15	3.45	0.80	1.01
9	2.18	0.32	0.64	15	3.27	0.75	0.98
9	2.18	0.38	0.70	15	2.73	0.50	0.80
9	2.64	0.49	0.79	15	6.95	1.99	1.59
9	2.27	0.36	0.68	15	2.19	0.34	0.66
9	2.00	0.27	0.59	15	2.67	0.38	0.70
9	1.64	0.20	0.50	15	2.95	0.60	0.87
9	2.05	0.29	0.61	16	3.91	0.95	1.10
9	2.81	0.52	0.81	16	2.00	0.29	0.61
9	2.64	0.48	0.78	17	4.48	0.75	0.98
9	2.71	0.29	0.61	17	4.38	0.88	1.06
9	1.71	0.21	0.52	19	4.00	0.87	1.05
9	2.10	0.33	0.65	19	3.33	0.63	0.90
9	2.29	0.35	0.67	21	3.95	1.00	1.13
9	2.48	0.49	0.79	22	5.27	1.78	1.51
9	3.05	0.56	0.84	22	8.27	3.89	2.23
9	1.91	0.29	0.61	22	5.81	1.59	1.42
9	1.91	0.24	0.55	24	7.86	2.28	1.70
10	1.91	0.25	0.56	24	6.43	2.03	1.61
10	1.55	0.17	0.47	25	8.29	3.43	2.09
10	2.64	0.44	0.75	25	5.57	1.89	1.55
10	1.91	0.28	0.60	29	8.96	4.06	2.27
10	3.18	0.43	0.74	31	4.64	1.36	1.32
10	1.81	0.28	0.60	38	6.62	2.84	1.90
10	2.27	0.34	0.66	44	7.71	3.89	1.7
10	2.48	0.40	0.71				

Table VIII Optic. nerve of undernourished rat, 180 days old.

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
4	0.86	0.04	0.23	8	1.09	0.09	0.34
4	2.14	0.33	0.65	8	1.68	0.2	0.53
4	1.05	0.06	0.28	8	1.09	0.09	0.34
5	1.45	0.15	0.44	8	1.14	0.09	0.34
5	1.82	0.17	0.47	8	1.27	0.11	0.37
5	4.09	0.72	0.96	8	1.09	0.10	0.36
6	0.50	0.02	0.16	8	1.32	0.12	0.39
6	1.41	0.17	0.47	8	1.41	0.13	0.40
6	0.82	0.06	0.28	8	0.91	0.06	0.28
6	1.73	0.23	0.54	8	1.41	0.13	0.41
6	0.91	0.05	0.25	8	1.55	0.17	0.26
6	1.41	0.13	0.41	8	2.45	0.40	0.71
6	0.95	0.05	0.25	8	1.05	0.08	0.32
6	1.77	0.18	0.48	8	0.77	0.04	0.23
6	2.64	0.21	0.52	8	1.73	0.20	0.50
6	1.18	0.09	0.34	8	2.59	0.47	0.77
6	1.64	0.21	0.5	8	1.55	0.15	0.44
6	3.64	0.57	0.85	9	1.27	0.13	0.41
6	1.86	0.20	0.50	9	1.50	0.16	0.45
6	1.27	0.11	0.37	9	1.73	0.20	0.50
6	1.09	0.08	0.32	9	1.95	0.26	0.58
7	1.32	0.14	0.42	9	.41	0.23	0.54
7	1.45	0.18	0.48	9	1.27	0.14	0.42
7	2.23	0.32	0.64	9	1.36	0.13	0.41
7	3.00	0.48	0.78	9	1.09	0.09	0.34
7	0.59	0.02	0.16	9	1.91	0.22	0.53
7	0.91	0.07	0.30	9	1.23	0.11	0.37
7	2.86	0.30	0.62	9	1.18	0.11	0.37
7	1.55	0.12	0.39	9	5.55	1.26	1.27
7	3.64	0.63	0.90	9	1.73	0.11	0.37
7	1.59	0.17	0.47	9	0.95	0.07	0.30
7	1.36	0.1	0.39	9	3.64	0.77	0.99
7	1.05	0.08	0.32	9	4.05	0.78	1.00
7	1.73	0.23	0.54	9	3.00	0.48	0.78
7	1.05	0.08	0.3	9	0.73	0.03	0.20
7	1.59	0.15	0.44	9	2.09	0.27	0.59
7	1.7	0.12	0.39	9	2.73	0.47	0.77
7	3.77	0.43	0.74	9	0.95	0.07	0.30
7	1.05	0.08	0.32	10	1.91	0.25	0.56
7	1.36	0.13	0.41	10	3.09	0.35	0.67
7	2.23	0.33	0.65	10	1.36	0.14	0.4
7	1.27	0.12	0.39	10	1.68	0.17	0.47
8	2.64	0.58	0.86	10	2.09	0.25	0.56
8	2.55	0.41	0.72	10	1.50	0.13	0.41
8	2.50	0.23	0.54	10	1.23	0.13	0.41
8	0.55	0.0	0.16	10	1.68	0.01	0.11
8	0.91	0.09	0.34	10	1.95	0.27	0.59
8	1.09	0.10	0.36	10	1.55	0.19	0.49
8	2.95	0.53	0.82	10	1.77	0.19	0.49
8	1.36	0.13	0.41	10	1.00	0.07	0.30
8	0.95	0.08	0.32	10	1.77	0.25	0.56
8	1.64	0.17	0.47	10	1.95	0.26	0.58

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
10	1.05	0.10	0.36	13	1.77	0.24	0.55
10	1.91	0.24	0.55	13	1.36	0.14	0.42
10	1.73	0.22	0.53	13	1.95	0.29	0.61
11	1.68	0.14	0.42	14	3.18	0.75	0.98
11	1.59	0.20	0.50	14	2.59	0.45	0.76
11	1.41	0.14	0.42	14	1.77	0.25	0.56
11	1.45	0.16	0.45	14	3.23	0.69	0.94
11	4.36	0.28	0.60	14	2.77	0.54	0.83
11	2.00	2.04	1.61	14	3.55	0.67	0.92
11	1.86	0.23	0.54	15	2.77	0.58	0.86
11	3.45	0.68	0.93	15	2.27	0.31	0.63
11	1.23	0.11	0.37	15	2.95	0.46	0.77
11	1.82	0.24	0.55	15	1.18	0.11	0.37
11	1.59	0.17	0.47	16	2.14	0.31	0.63
11	1.73	0.22	0.53	16	3.45	0.62	0.89
11	2.23	0.36	0.68	16	4.05	0.92	1.08
11	1.64	0.21	0.52	16	6.00	0.73	0.96
11	2.14	0.24	0.55	16	2.00	0.30	0.62
11	3.59	0.87	1.05	16	5.45	1.48	1.37
11	1.95	0.26	0.58	17	2.59	0.47	0.77
11	1.73	0.23	0.54	17	2.45	0.26	0.58
12	1.90	0.24	0.55	18	8.55	2.59	1.82
12	3.82	0.94	1.09	21	4.50	1.25	1.26
12	1.64	0.21	0.52	22	3.91	1.00	1.13
12	1.00	0.08	0.32	23	6.09	1.57	1.41
12	1.77	0.22	0.53	24	4.91	1.56	1.41
12	0.91	0.06	0.28	29	6.41	2.23	1.69
12	2.68	0.50	0.80	30	7.86	2.37	1.74
12	2.14	0.33	0.65	32	6.68	2.16	1.66
12	1.18	0.10	0.36	32	7.18	2.36	1.73
12	1.55	0.18	0.48	34	7.14	2.74	1.87
12	1.95	0.28	0.60	34	11.55	4.14	2.30

Table IX. Optic nerve of rehabilitated rat, 180 days old.

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
5	1.30	0.13	0.41	7	2.13	0.33	0.65
5	1.83	0.26	0.58	7	1.04	0.07	0.30
5	1.22	0.11	0.36	7	0.61	0.03	0.20
5	2.00	0.33	0.65	7	1.09	0.10	0.36
5	1.57	0.20	0.50	7	2.17	0.40	0.71
5	1.48	0.17	0.47	7	2.17	0.36	0.69
5	1.48	0.16	0.45	7	1.57	0.19	0.49
6	2.26	0.32	0.64	8	0.87	0.06	0.28
6	1.65	0.20	0.50	8	1.91	0.26	0.58
6	1.70	0.26	0.58	8	3.04	0.49	0.79
7	1.83	0.29	0.61	8	1.83	0.27	0.59
7	1.83	0.26	0.58	8	2.00	0.28	0.60
7	1.48	0.17	0.47	8	1.39	0.14	0.4

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
8	1.30	0.14	0.42	11	1.65	0.23	0.54
8	1.83	0.27	0.59	11	2.26	0.39	0.70
8	1.65	0.20	0.50	11	2.43	0.41	0.72
8	2.00	0.33	0.65	11	1.48	0.17	0.47
8	1.65	0.19	0.49	11	1.87	0.27	0.59
8	1.91	0.26	0.58	11	2.35	0.44	0.75
8	2.09	0.33	0.65	12	1.83	0.26	0.58
8	1.65	0.21	0.5	12	1.83	0.20	0.50
8	1.83	0.23	0.54	12	2.57	0.13	0.41
8	0.87	0.06	0.28	12	2.74	0.50	0.80
8	1.30	0.13	0.41	12	2.52	0.45	0.76
8	2.22	0.38	0.70	12	3.04	0.62	0.89
8	1.74	0.23	0.54	12	1.83	0.34	0.66
8	1.39	0.16	0.45	12	1.57	0.19	0.49
8	1.57	0.21	0.52	12	1.48	0.10	0.50
8	1.74	0.23	0.54	12	2.17	0.38	0.70
8	1.35	0.15	0.44	12	1.74	0.23	0.54
8	1.57	0.20	0.50	12	2.61	0.4	0.73
9	1.91	0.27	0.59	13	2.26	0.33	0.65
9	1.35	0.13	0.41	13	2.70	0.43	0.74
9	1.83	0.20	0.50	13	2.61	0.53	0.82
9	1.87	0.24	0.55	13	1.74	0.22	0.53
9	1.91	0.29	0.61	13	1.96	0.31	0.63
9	1.52	0.18	0.48	13	1.65	0.19	0.49
9	2.00	0.31	0.63	13	2.61	0.40	0.71
9	1.65	0.23	0.54	13	2.61	0.55	0.84
9	1.57	0.18	0.48	14	2.17	0.38	0.70
9	1.65	0.23	0.54	14	2.44	0.44	0.75
9	1.87	0.12	0.39	14	2.61	0.51	0.80
9	2.26	0.35	0.67	14	2.52	0.47	0.77
9	1.57	0.20	0.50	14	2.52	0.50	0.80
9	1.61	0.21	0.52	14	1.65	0.18	0.48
9	1.22	0.14	0.42	14	2.09	0.33	0.65
9	1.96	0.29	0.61	15	3.16	0.66	0.92
9	1.74	0.22	0.53	15	2.35	0.32	0.64
9	1.57	0.22	0.53	16	1.83	0.26	0.58
9	1.65	0.15	0.44	17	3.30	0.66	0.92
9	1.87	0.6	0.58	17	2.48	0.84	1.03
9	1.52	0.10	0.30	18	2.91	0.68	0.93
9	1.2	0.11	0.37	18	3.70	1.10	1.18
10	2.09	0.29	0.61	18	3.91	1.07	1.17
10	3.09	0.53	0.82	18	2.52	0.39	0.70
10	1.78	0.25	0.56	19	2.57	0.53	0.82
10	1.91	0.34	0.55	20	2.96	0.66	0.92
10	1.74	0.6	0.58	20	2.57	0.99	1.12
10	1.83	0.4	0.55	21	3.39	0.86	1.05
10	1.74	0.22	0.53	21	4.09	1.00	1.13
10	1.22	0.22	0.53	22	2.70	0.53	0.82
10	1.43	0.14	0.42	24	3.04	0.74	0.97
10	1.83	0.0	0.50	24	4.00	1.17	1.22
10	2.48	0.45	0.76	25	4.96	1.17	1.22
11	1.74	0.23	0.54	28	5.83	.66	1.84
11	2.48	0.43	0.74	31	5.22	1.76	1.50

Number of lamellae	Circumference μ	Area μ^2	Diameter μ	Number of lamellae	Circumference μ	Area μ^2	Diameter μ
31	5.39	1.85	1.54	36	8.09	4.07	2.28
35	4.87	1.77	1.50	40	9.13	5.73	2.70
35	11.48	3.03	1.96	44	5.65	2.16	1.66
35	6.09	2.39	1.74	46	6.26	2.81	1.89

Table X. Lamellar counts of the ventral root

Rat and Age in days	Groups of pooled lamellae														Number of lamellae measured
	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	101-110	111-120	121-130	131-140	
Control 90 days		1	6	14	15	11	8	13	10	18	18	15	31	8	168
Under-nourished 90 days		1	9	26	22	34	20	13	5	12	17	32	7		198
Rehabilitated 90 days				18	18	21	10	15	16	31	25	22	15	8	199

Table XI Lamellar counts of the dorsal root

Rat and Age in days	Groups of pooled lamellae														Number of lamellae measured
	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	101-110	111-120	121-130	131-140	
Control 90 days		2	7	27	27	20	11	20	18	30	38	10	6	1	228
Under-nourished 90 days		1	9	21	42	26	29	21	23	26	21	16	15	7	245
Rehabilitated 90 days		8	19	51	36	31	18	16	21	31	15	11	9		260

Table XII Lamellar counts of the optic nerve

Rat and Age in days	Groups of pooled lamellae										Number of lamellae measured
	0-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	
Control											
180 days	10	162	127	50	21	8	9	3	2		392
Under nourished											
180 days	6	241	139	36	6	3	11				437
Rehabili- tated											
180 days	10	162	134	52	18	9	7	6	2	1	401

ACTA PHYSIOLOGICA SCANDINAVICA
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BLOOD FLOW**

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FROM THE DEPARTMENT OF CLINICAL PHYSIOLOGY
KAROLINSKA Sjukhuset
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The present thesis is based on the following papers

- I Eklund B and L Kaijser Forearm blood flow after isometric contraction at different loads in relation to potentially vasodilating substances
Scand J clin Lab Invest 1974 Accepted for publication
- II Kaijser L and B Eklund The effect of nor-adrenaline and of increased sympathetic activity on the hyperaemia following short and prolonged forearm work
Scand J clin Lab Invest 33 1974 in press
- III Eklund B and L Kaijser The effect of close arterial infusion of isoproterenol and phentolamine on the blood flow following short-term and prolonged work
Res Commun Chem Pathol Pharmacol 1974 Accepted for publication
- IV Eklund B L Kaijser and E Knutsson Blood flow in resting arm and leg during contralateral isometric contraction *J Physiol (Lond)* 1974 in press
- V Eklund B Vascular response to hyperosmotic stimulation during and after prolonged and short work
This supplement Part B
- VI Eklund B Modification of the forearm blood flow response to contralateral isometric hand-grip by prolonged and short work
This supplement Part B

The papers will be referred to by their Roman numbers

Introduction

PREVALENT OPINIONS CONCERNING BLOOD FLOW REGULATION
IN EXERCISING MUSCLE

The increase in blood flow in the exercising skeletal muscle has been studied extensively for many decades and the factors that adapt the muscle blood flow to work intensity and oxygen demand have been subject to much debate. The conception that local factors such as chemical or physical changes linked to the metabolism of the muscle produce exercise hyperaemia was suggested already by Gaskell (1880) and that they play the most important role in its maintenance seems now generally accepted.

At rest the tone of the precapillary resistance and sphincter sections in the vascular bed of the skeletal muscle is considered to be regulated mainly by an intrinsic myogenic activity facilitated by the distention of contractile elements of the vascular wall (Bayliss 1902, Folkow 1949, Folkow and Öberg 1961). Superimposed on this local regulation of the precapillary vessels is a central vasomotor control which in man is mediated by sympathetic adrenergic fibres (for references see Folkow 1955 and Uvnäs 1960 and 1967). During exercise vasodilating metabolites released from the active muscles overcome to a great extent the myogenic activity as well as the constrictor fibre influence on the precapillary vessels of the muscle while the nervous influence on the postcapillary section is still maintained (Kjellmer 1964 and 1965 a, Strandell and Shepherd 1967).

A large number of different substances produced locally during muscle contraction have, alone or in combination, been suggested as mediators of the increase in muscle blood flow during exercise. Histamine was discussed early (Anrep and Barsoum 1935) but its participation in the production of exercise hyperaemia was not confirmed (Fleisch and Weger 1937, Emmelin, Kahlson and Wiksell 1941) the same applying to bradykinin (Hilton and Lewis 1958, Kjellmer and Odellram 1965). Lactic and pyruvic acids are produced by the exercising muscle and Molnar et al (1962) investigated the local vascular action of these acids in the canine forelimb. Intra-arterial infusion caused a moderate decrease in vascular resistance which was attributed to the concomitant increase in H^+ ions since it was not observed when sodium lactate or pyruvate was infused. Furthermore in McArdle's syndrome lactate is not produced by the active muscle.

but still muscle blood flow increases markedly during work (Pernow Havel and Jennings 1967) However if not a very potent vasodilator in itself lactate is probably one of the more important substances contributing to the increase in osmolality (Lundvall 1972 Pernow personal comm see below) CO_2 is also produced in the working muscle and the effect of CO_2 has been evaluated by Kontos and co-workers (Kontos and Patterson 1966 Kontos Richardson and Patterson 1967 Kontos 1971) in animal as well as human studies They conclude that local hypercapnia plays only a minor role in the production of hyperaemia in the contracting muscle

Adenosine may according to Berne et al (1971) be involved in the local regulation of skeletal muscle blood flow but is probably more important in the regulation of coronary blood flow (Rubio and Berne 1969) ATP has been identified in the effluent venous blood from ischemic working muscle (Forrester and Lind 1969 Forrester 1972) and since it has a pronounced vasodilator action it is suggested to be involved in the local regulation of flow Hilton (1971) suggested that inorganic phosphate may also be a mediator of exercise hyperaemia Barcroft and co-workers (1971) however were not able to produce any substantial increase in forearm blood flow by *intraarterial infusions of inorganic phosphate in amounts that raised the level of phosphate in the effluent venous blood to values far above those seen during exercise*

Hypoxia in the wall of the resistance vessels has been suggested by Carrier Walker and Guyton (1964) and Detar and Bohr (1968) as a possible cause of the vasodilatation during exercise but there seems to be little reason to believe that the oxygen tension to which the resistance section of the vessels is exposed decreases sufficiently during work under normal conditions Duling and Berne (1971) have indeed shown that the oxygen tension on the external surface of the artery starts to decrease to a certain extent already between the larger artery and the terminal artery Such an effect must however play a minor role since on the venous side of the muscle vascular bed the PO_2 decreases rather little with an increased work load suggesting only a moderate decrease in PO_2 at tissue level at the same time as blood flow increases severalfold (Wahren 1966 Kaijser 1970)

Potassium ions are released from contracting muscle cells and Kjellmer (1965 b) proposed that the increase in potassium ions in the extracellular fluid can account for a considerable part of the vasodilatation However since plasma concentration of potassium is rapid-

ly normalized after work (Laurell and Parnow 1966) It can thus hardly be responsible for the prolonged hyperaemia following exercise Skinner and Costin (1970 1971) have studied the interaction between oxygen lack and increase in potassium concentration and osmolality and concluded that these factors reinforce one another in lowering the resistance of the canine muscle vascular bed

Most authors agree that probably several factors jointly produce the vasodilatation in the working muscle An attempt to explain the nature of such a joint effect was made by Mellander and co-workers (Mellander et al 1967 Lundvall Mellander and White 1969 Mellander and Lundvall 1971 Lundvall 1972), who suggested hyperosmolality induced by the local formation of several metabolites during exercise to be the essential vasodilating factor in exercise hyperaemia In reviewing the literature Mellander and Johansson (1968) also stated the criteria some of which are given below that should be fulfilled for a given metabolic factor if it is to be accepted as of importance in mediating exercise hyperaemia 1) The substance should belong to the group of agents naturally occurring in muscle tissue It should be demonstrable in the tissue or the effluent venous blood during exercise and the concentration of the substance should bear some quantitative relationship to the degree of vasodilatation 2) Intraarterial administration of the substance in amounts comparable to those released during exercise should in resting muscles give the same magnitude and pattern of vascular response as that of exercise itself 3) The mode of elimination of a dilator factor after infusion should be compatible with the approximately exponential decrease of flow seen in the postexercise period

A major part of the studies on local chemical or physical changes that might influence vascular resistance in working muscles has been performed as animal experiments where muscle contraction has been accomplished by nerve stimulation or on isolated blood vessels which for technical reasons must be fairly large and hence not representative of the terminal arteries involved in resistance regulation (Burton 1954) Electrically induced muscle activity might well differ from muscle activity in man performing exercise with regard to metabolite release Thus forearm muscle activity induced by electrical stimulation seems to result in a greater release of lactate at a given oxygen uptake level than a corresponding voluntarily induced rhythmic dynamic activity (Landin and Wahren

1968) Studies in man on physiological modes of exercise are relatively few Barcroft (1971) Kontos and co-workers (1966, 1967) and Forrester and Lind (1969) undertook their studies on the human forearm (see above) Lundvall et al (1969) compared the effects of forearm exercise and close Intraarterial Infusion of hypertonic solutions on blood flow in man in order to establish a quantitative relationship between forearm blood flow and osmolality A hypertonic infusion producing an increase in venous osmolality comparable to that found during exercise did not decrease vascular resistance to the same degree but the authors ascribed the discrepancy to the possibility that muscle activity increased tissue osmolality more than infusion

Most of the studies here referred to have not taken into account the effect of work duration on muscle metabolism and hence its possible influence on flow adaptation by local factors Often the work duration is imprecisely stated but the majority of the studies refer to short periods of muscular activity Ekelund (1967) found that once a relative hemodynamic steady state was established cardiac output remained largely unchanged during bicycle exercise for one hour Jorfeldt (1970) and Wahren and Hagenfeldt (1968) studied the local circulation in the human forearm during dynamic work of the same duration Blood flow was found to increase slightly with the duration of exercise while oxygen uptake remained constant Thus oxygen saturation and tension of the effluent venous blood was increased Furthermore as has been shown earlier (Bang 1936) the lactate concentration in the effluent venous blood decreased with time which is also the case in the muscle tissue (Saltin and Karlsson 1970) and PCO_2 and H^+ were concomitantly lowered Thus some of the chemical changes in the effluent venous blood and muscle tissue are more pronounced during the initial phase of exercise than at the end of a prolonged work period and still blood flow is maintained at an approximately steady level

Aim of the study The relative Importance of the different possible mediators of the Increased local blood flow during exercise has not been agreed upon Furthermore studies have rarely been performed under conditions of well defined modes and durations of work Therefore the aim of the present investigations was

1 Further to study the relation of blood flow to some of the factors that have been ascribed major importance in the production of exercise hyperaemia in connection with short-term muscle activity

2 To gain more information concerning the Importance of different flow promoting factors by studying the relation between flow and metabolite concentration in the effluent venous blood during work of different durations yielding different metabolite release

3 To Investigate whether prolonged work induces a change in sensitivity of the muscle vascular bed to vasoactive stimulation which might be of importance for blood flow maintenance since the literature indicates that prolonged work changes the proportion between blood flow and the concentration of some metabolites in the effluent venous blood

The intention was to study the influence of work duration on the relationship between blood flow and metabolites as well as on the sensitivity of the vascular bed both during and shortly after work of different durations One motive for performing studies on vascular reactivity also shortly after work was that studies of the response to vasoactive stimulation require a method that can measure rapid changes in blood flow The only method satisfying this requirement possible to use in human studies seems to be venous occlusion plethysmography a method which however with few exceptions can be applied only on a resting limb

To follow and quantify rapid changes in flow also during work the deep venous O_2 saturation was used as indicator of flow changes

Chapter 1

GENERAL PROCEDURES AND METHODS

Subject In all 72 healthy male volunteers of ordinary physical fitness aged 20-54 years (mean age 32) were studied. Several of them took part in more than one of the series of experiments.

Modes of exercise Exercise was performed with the forearm.

Two modes of exercise have been used.

1 Sustained isometric handgrip contraction performed on a dynamometer with immovable handles (Flygtekniska Försöksanstalten, Stockholm. In some experiments a similar dynamometer manufactured by Bofors AB, Karlskoga was used). The force developed was measured by a strain gauge and displayed to the subject on an oscilloscope or by the pointer of a galvanometer.

2 Rhythmic dynamic forearm work performed on a spring loaded hand ergometer with a contraction path of 40 mm. Contraction frequency was 60/min, governed by a metronome. The load was determined by the length and number of the springs (Kaijser 1970).

The exercise was performed in the recumbent position with the forearm at heart level. The isometric contraction was performed with the elbow in a slightly flexed position and the dynamometer in a vertical position. The dynamic work with the elbow extended and the dorsal side of the hand facing upwards.

Catheterization procedures A brachial artery was catheterized percutaneously for blood pressure recording or the infusion of vasoactive substances. Teflon catheters were used with an outer diameter of 1.4 mm and the tip adapted to a guide line with a diameter of 0.9 mm. For the infusion of vasoactive substances the catheter was introduced about 10 cm into the brachial artery of the arm to be studied. For arterial blood pressure recording the contralateral arm was catheterized.

The same type of teflon catheter was used for the catheterization of the deep venous system of the forearm and was introduced into a cubital vein in the distal direction and manipulated into a deep vein so that the tip could not be palpated. The distance it had been introduced 4-10 cm was measured and checked again when the catheter was withdrawn after the termination of the

experiment With the catheter in this position it is possible to sample blood draining muscle tissue almost exclusively during exercise and also at rest if the circulation of the hand is occluded (Cooper Edholm and Mottram 1955 Coles et al 1958 Idbohrn and Wahren 1964)

Intraarterial infusions of vasoactive substances were performed with a motordriven constant speed pump

Forearm blood flow measurement Blood flow in the forearm was measured by venous occlusion plethysmography The plethysmograph consisted of an airfilled rubber cuff according to Dohn (1956) and Graf and Westersten (1959) The plethysmograph cuff was placed around the thickest part of the forearm the enclosed segment being 5 cm long The cuff was inflated to about 40 mm H₂O Volume changes in the forearm were recorded as pressure changes in the plethysmograph and calibrated with a known volume of air The venous occlusion pressure was 50 mm Hg During the recording of inflow curves the circulation of the hand was occluded by a wrist cuff inflated to 240 mm Hg The pressure increase in the plethysmograph following venous occlusion was measured with a pressure transducer (EMT 33 Siemens-Elema) and recorded on an ink jet recorder (Mingograf 34 or 81 Siemens-Elema) Blood flow is expressed in ml/min $\times 100$ ml of tissue

Skin and muscle temperatures Skin temperature was measured with a Tele-thermometer (Yellow Springs Instrument Inc) the probe taped to the skin immediately distal to the plethysmograph cuff Muscle temperature was measured with a thermocouple situated at the tip of a thin polyethylene catheter (Ellab Instruments) introduced percutaneously into the brachio-radialis to a depth of 20-25 mm beneath the skin under the plethysmograph cuff

Arterial pressure was measured with a capacitance transducer (EMT 34 or 35 Siemens-Elema) and recorded on a Mingograf 81 (Siemens-Elema) Mean pressure was obtained by electrical integration time constant 1 sec or in the studies presented in Chapter 6 3 sec

Heart rate was obtained from ECG recordings

Blood analyses Blood samples for the determination of O₂ saturation PO₂ PCO₂ and pH were drawn into siliconized glass syringes where the dead space was filled with he-

Calculations Resting or basal forearm blood flow was calculated from at least five inflow curves. In the study presented in Chapter 2 (Paper I) the average flow during the first 30 sec period after contraction was calculated for each subject from all inflow curves recorded during this interval and the average flow for the second 30 sec period was calculated similarly. At later intervals flow was calculated from four to six successive inflow curves. In the series of experiments comparing short and prolonged work, the first, second and third inflow curves after the 5 and the 55 min work period were averaged separately for the group of subjects. At other times inflow curves recorded during a 30 sec period were averaged for the individual subject to represent the flow at that time. When flow changes were rapid, blood flow values are given for 15 sec intervals and are then calculated from one or two inflow curves. Maximal blood flow, e.g. during infusion of vasodilating substances or during contralateral isometric contraction is calculated as the mean of the two steepest successive inflow curves. Forearm vascular resistance is calculated as mean arterial pressure divided by forearm blood flow

$$\frac{\text{mm Hg}}{\text{ml} \times \text{min}^{-1} \times 100 \text{ ml}^{-1}}$$

Statistical methods Statistical calculations have been performed according to conventional methods (Snedecor 1959). When not otherwise stated values given in the text and tables are mean values \pm standard error of the mean for the group studied. Significance tests performed on values obtained from a group of subjects at different times have been calculated by paired comparisons.

Error of methods The error of the methods used for the various blood analyses are given in Table 1. In the series of experiments presented in Chapter 5 the osmolality of each blood sample was determined four times. The error of the method was then 0.42 per cent of the mean value. The error of the forearm blood flow measurements includes biological variations as well as the methodological errors. The error of a single determination in per cent of the mean value as estimated from duplicate determinations of resting blood flow was 10.1 per cent (mean $4.33 \text{ ml} \times \text{min}^{-1} \times 100 \text{ ml}^{-1}$, number of duplicate determinations 39). At increased flow rates produced by con-

tinuous prolonged Intraarterial infusion of phentolamine the error was 16.7 per cent (mean value $8.43 \text{ ml} \times \text{min}^{-1} \times 100 \text{ ml}^{-1}$ $n=32$). The reproducibility calculated from blood flow determinations at corresponding intervals after two identical periods of submaximal dynamic forearm work of 15 min duration separated by 90 min rest, was 12.0 per cent of the mean value (mean $25.5 \text{ ml} \times \text{min}^{-1} \times 100 \text{ ml}^{-1}$ $n=22$).

Definitions In the present publication 'short-term work' refers to durations of approximately 5 min and 'prolonged work' to durations of approximately 1 hour.

For convenience the term 'metabolites' is used to denote all substances released from the active muscle.

Table 1 Errors of a single determination as estimated from duplicate determinations

$$E = \sqrt{\frac{\sum d^2}{2n}}$$

Variable	Range	Number of duplicate determinat	Mean value	Error	Error in per cent of mean value
O ₂ saturation % according to Drabkin (1950)	10-40 40-90	36 51	30.9 57.1	0.34 0.48	0.10 0.84
CO-Oximeter(Instr Lab)	30-90	26	59.5	0.86	1.45
PO ₂ mm Hg	40-100	25	66.8	2.29	3.43
PCO ₂ mm Hg	20-60	25	30.5	0.93	3.08
pH	7.20-7.50	25	7.425	0.009	1.21
Lactate mmol/l	0-2 2-8	36 32	1.37 3.63	0.06 0.13	4.3 3.8
Pyruvate mmol/l	0-0.10 0.10-0.30	35 32	0.072 0.132	0.006 0.005	8.3 3.7
Na ⁺ mEq/l	125-145	20	132.1	0.177	0.13
K ⁺ mEq/l	2.0-5.0	20	3.81	0.016	0.40
Osmolality mosm/kg	260-310	50	276.7	1.103	0.58

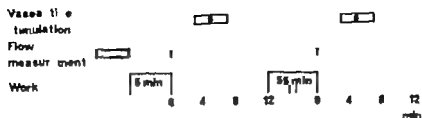


Fig 1 General design of experiments comparing the the effect of vasoactive stimulation on postexercise forearm blood flow after short term (5 min) and prolonged (55 min) work



Fig 2 General design of experiments comparing the effect of vasoactive stimulation on deep venous oxygen saturation during short term and prolonged work

Chapter 2

RELATIONSHIP BETWEEN BLOOD FLOW AND RELEASE OF METABOLITES AFTER SHORT-TERM ISOMETRIC MUSCLE ACTIVITY (Paper 1)

As an introduction to the studies of relations between blood flow and metabolites during work of different durations the effect of short-term forearm muscle activity of defined intensities on forearm blood flow and on the concentration of vasoactive substances in the effluent venous blood was investigated. Muscle activity was performed as isometric contraction. Under these conditions the mechanical effect of the contraction reduces the blood flow substantially (Humphreys and Lind 1963, Edwards, Hill and McDonnell 1972). Consequently metabolites can be assumed to accumulate and blood and tissue concentrations of metabolites might tend to equilibrate towards the end of the contraction period.

Procedure Seven subjects were studied performing isometric handgrip at $1/3$, $1/4$ and $1/8$ MVC for 2 minutes. $1/3$ MVC is slightly below the load leading to fatigue in 2 minutes. Resting and post-contraction forearm blood flow was measured plethysmographically. Deep venous blood was sampled at rest, at the end of and after the contractions.

Results and Discussion Conclusions regarding tissue concentrations of substances drawn from their concentrations in the effluent venous blood from isometrically contracting muscle are uncertain. If the blood flow through the muscle is partly hindered, the flow is small in relation to the metabolic rate and hence blood and tissue concentrations might tend to equilibrate towards the end of the contraction period. In that case the immediate post-contraction blood flow would be better correlated with the blood concentrations of flow-promoting metabolites at the end of the contraction than those during the hyperaemic phase after it. If however blood flow through the contracting muscle is completely arrested, there may be an equilibration only of a limited amount of capillary blood and this would attenuate the changes in the venous blood sampled during contraction.

The above mentioned circumstances must be considered when conclusions regarding cause and effect are drawn from relationships found between blood flow and

metabolite concentrations in the effluent venous blood of the muscle

Contraction at $1/8$ MVC increased forearm blood flow five times the resting level; contractions at $1/4$ and $1/3$ MVC increased the flow further roughly in proportion to the loads. The post-contraction blood flows differed significantly between the loads still at three minutes after the contractions.

Lactate and pyruvate concentration as well as osmolality increased during contraction to a higher degree with higher load. Of these, pyruvate concentration decreased immediately after contraction and then again increased; thus the changes do not parallel the changes in blood flow. Lactate showed a tendency to increase further after contraction and to remain elevated while the blood flow decreased more rapidly. This would suggest that lactate concentration is not a specific flow-promoting factor. However, it may well be one of the more important substances contributing to the increase in osmolality. Osmolality increased in five of the subjects at the two lower loads, but the change for the group did not reach significance. At the highest load it increased by 23 mosm/l . Thus the quantitative relationship between peak flow and osmolality was not quite convincing. However, the decrease in osmolality rather well paralleled the decrease in blood flow, indicating that osmolality may be of importance as a flow-promoting factor as suggested by Lundvall, Mellander and White 1969 and Lundvall 1972.

PCO_2 and acidity increased very little during, but markedly after contraction, rendering the difference between the loads largest immediately after contraction. If the contention is valid that blood flow is arrested by the contraction and the blood sampled after contraction reflects tissue concentrations better than that sampled during it, these two factors show a relation to blood flow which might indicate that they contribute to the post-contraction hyperaemia.

PO_2 decreased slightly towards the end of the contractions but did not differ between the loads. Increased immediately after contractions and furthermore reached the highest level after the heaviest load. It therefore seems justifiable to rule out low O_2 tension during or immediately after contraction as a flow-promoting factor under physiological conditions. The K^+ concentration increased during contraction but to the same extent at the different loads. Furthermore the concentration decreased again immediately after contraction which is in accordance with

the findings of Laurell and Pernow (1966) and thus it does not seem likely that K^+ contributes to the hyperaemia after work.

Conclusion Of the different metabolic factors studied osmolality was best correlated with the blood flow pattern after isometric muscle activity. Lactate was probably the important substance causing the changes in osmolality. Furthermore the data are in accordance with the contention that CO_2 and H^+ to a minor degree might contribute to the vasodilatation.

Chapter 3

BLOOD FLOW AND RELEASE OF METABOLITES FROM EXERCISING MUSCLES DURING AND AFTER PROLONGED AND SHORT WORK (Paper II)

The regulation of muscle blood flow during exercise and the possible influence on it of local metabolic factors have mainly been studied in connection with relatively short periods of work without consideration of the effect of work duration on muscle metabolism and thereby eventually on blood flow adaptation. The blood concentration of lactate decreases with work duration (Bang 1936) and during forearm work of one hour also the H^+ concentration and PCO_2 of the effluent venous blood is lower than during short work while muscle blood flow is slightly greater (Wahren and Hagenfeldt 1968, Jorfeldt 1970).

In the present series of experiments post-exercise forearm blood flow was recorded after short and prolonged dynamic forearm work in order to gain information regarding possible differences in spontaneous course of flow decrease which might suggest different mechanisms for the maintenance of the hyperaemia. The concentration of some conceivably vasoactive substances was also studied in the effluent venous blood during and after the short and the prolonged work to establish their relation to the blood flow.

Procedure In 14 subjects performing dynamic forearm work at an average load of 7.5 kpm/min for 5 and 55 min forearm blood flow was recorded plethysmographically for 12 min after the work periods without inserted catheters or other interventions. In 20 additional subjects a deep vein was catheterized percutaneously for blood sampling at rest as well as during and after the short and the prolonged work. Skin and muscle temperatures were recorded in all subjects.

Results Forearm blood flow was the same immediately after the short and the prolonged work. After the short work flow decreased rapidly at first and then more slowly towards resting level. After the prolonged work the decrease during the first minute was even more rapid but from the second minute after work no further decrease took place during the rest of the measurement period blood flow remaining at rest level 3.4 times the resting

flow and hence also above the flow at corresponding times after the short work

PO_2 in the deep vein was significantly higher and PCO_2 and H^+ concentration lower at the end of the prolonged than at the end of the short work period. Also lactate and pyruvate concentrations were lower at the end of the prolonged than the short work. During short-term work K^+ concentration and osmolality increased slightly but significantly but at the end of prolonged work it had decreased again to the resting level. The Na^+ concentration was not at all influenced by exercise.

Skin and muscle temperatures increased about $1^\circ C$ during the short and further about $1.5^\circ C$ during the prolonged work. During the 12 min period of blood flow recording after the work periods they remained essentially unchanged.

Discussion As evident from the present and earlier studies (Wahren and Hagenfeldt 1968, Jorfeldt 1970) the forearm blood flow during and immediately after prolonged work is slightly larger than or the same as that during and after short-term work. However, the venous blood concentration of several substances linked to the metabolism of the muscle such as CO_2 , H^+ , K^+ and lactate as well as osmolality which were markedly increased during the short work had decreased significantly again at the end of the prolonged work. Thus, the local blood flow in working muscle can hardly be regulated by the simple effect of any of these factors mentioned above. Theoretically however they may still be of importance either if the sensitivity of the vascular bed to these substances is increased or if the vasoconstrictor effects which they have to counteract are decreased.

The time course of disappearance of the hyperaemia differed after prolonged and short work. Thus after prolonged work the flow decreased more rapidly at first and then remained unchanged at a level 3-4 times the resting and hence also higher than the flow at corresponding times after short-term work. This altered spontaneous rate of flow decrease might indicate that partly different mechanisms maintain the hyperaemia after and possibly also during prolonged work.

Tissue temperature seems to be the only variable determined in this study which is higher after prolonged work and might thus be partly responsible for the difference in flow and taken into consideration as a possible factor contributing to maintain muscle blood flow during prolonged work.

Chapter 4

VASCULAR RESPONSE TO ADRENERGIC STIMULATION DURING AND AFTER PROLONGED AND SHORT WORK (Papers II and III)

Since the concentration of several vasodilator metabolites in the effluent venous blood from the exercising muscle decreases progressively during prolonged work while the blood flow is maintained at a steady level (Paper II) it must be assumed either that their importance in the regulation of local blood flow is diminished or that either the sensitivity to such metabolites has increased or the vasoconstrictor effects which they have to counteract are decreased by prolonged work. In the series of experiments presented in this chapter the possibility of an altered response to adrenergic stimulation induced by prolonged work was investigated. The following experimental situations were studied

1 Response to adrenergic vasoconstriction after as well as during short and prolonged work both as the effect of close intraarterial infusion of noradrenaline and as the effect of increased sympathetic nervous activity produced by the application of subatmospheric pressure to the lower part of the body (lower body low pressure (LBLP)). For the study of vascular response to adrenergic stimulation during exercise changes in deep venous oxygen saturation was used as an indicator of blood flow changes with the assumptions previously discussed (Chapter I)

2 Modification of the response to infusion of noradrenaline after short and prolonged work by local β -adrenergic blockade

3 Effect on blood flow of β -adrenergic stimulation by close intraarterial infusion of isoproterenol after short and prolonged work

4 Effect of α adrenergic blockade by close intraarterial infusion of phentolamine after short and prolonged work

Procedures In all 61 subjects took part in the investigation performing dynamic forearm work at a load of 7-8 kpm/min. In the studies performed during work 0.2 μ g noradrenaline per minute was infused intraarterially or LBLP (10-30 mm Hg below atmospheric) was applied for 3 min early during the continuous work period

and again towards the end of it and deep venous blood was sampled as shown in Fig 2 (Chapter 1)

Vasoactive stimulation by LBLP or infusion of drugs shortly after work was performed as shown in Fig 1 (Chapter 1) during the 12 min period of plethysmographic blood flow recording. Noradrenaline was infused in a dose of 0.1 $\mu\text{g}/\text{min}$ isoproterenol in doses of 0.02-0.08 $\mu\text{g}/\text{min}$ and phentolamine in doses of 150-200 $\mu\text{g}/\text{min}$. Regional β -adrenergic blockade was obtained by the infusion of 0.5 mg propranolol during the last minute of the work periods (Johnsson 1967)

Results When noradrenaline was infused during the continuous work period the decrease in deep venous O_2 saturation was more pronounced after 60 than after 4 min of forearm work indicating a more pronounced flow reduction (Fig 3). LBLP however induced the same decrease in deep venous O_2 saturation when applied at 4 and 60 min (Fig 4).

When applied shortly after the work periods noradrenaline and LBLP produced a less pronounced decrease in forearm blood flow after the prolonged than after the short work but this difference is probably accounted for by the difference in spontaneous rate of flow decrease (Paper II). After the prolonged work however noradrenaline infusion was followed by a marked flow increase above the preinfusion level which was seen neither after short work nor on the termination of LBLP in the same situation.

Regional β -adrenergic blockade did not significantly influence the flow decrease during noradrenaline infusion but abolished the increase in flow above preinfusion level on the termination of infusion after prolonged work.

Isoproterenol infusion after the short work increased the forearm blood flow to a value more than twice the preinfusion flow. The flow decreased again but remained above the preinfusion value during the whole infusion period. After prolonged work isoproterenol increased flow by 50 per cent which is significantly less than after the short work.

Phentolamine increased forearm blood flow only slightly by about 15 per cent when infused after the short work. After the prolonged work the increase was significantly larger being nearly 60 per cent.

Discussion The findings have provided some indications that prolonged work might increase the response of the forearm vascular bed to α - as well as β -adrenergic effects.

of infused noradrenaline. In contrast no difference in response to increased sympathetic nerve activity could be discerned. The observation that the large increase in blood flow on the termination of noradrenaline infusion after prolonged work could be abolished by β -adrenergic blockade initiated the study of the response to β -adrenergic stimulation by isoproterenol. The flow increase in response to infusion of isoproterenol however was less pronounced after prolonged work and thus not in accordance with the hypothesis that prolonged work might induce a greater sensitivity to β -adrenergic stimulation. However there might be differences in response to a bloodborne β -adrenergic stimulating agent and the transmitter released from the nerve terminal. The response to an α -adrenergic blocking agent phentolamine was more marked after prolonged work. This finding might imply that α -adrenergic stimulation or the sensitivity to such stimulation, is more pronounced after prolonged work. However the latter explanation seems to be excluded by the earlier described observations. An additional explanation would be that the sensitivity to the non-adrenergic vasodilator effect which has been ascribed to phentolamine (Taylor et al 1965 a, b and c) is more pronounced after prolonged work.

Conclusion The quantitative response to increased adrenergic nervous vasoconstrictor stimulation seems not to be altered to a significant degree by prolonged work which however induces a qualitatively altered response to circulating noradrenaline which might be due to a more long-lasting β -adrenergic stimulation. Phentolamine caused a greater blood flow increase after prolonged than after short work probably due to an increased sensitivity to the direct vasodilator action of the drug.

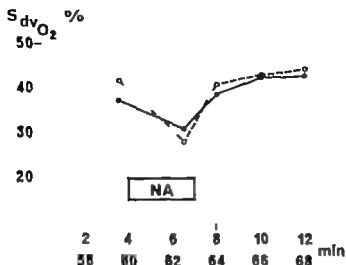


Figure 3 Effect of Infusion of noradrenaline on deep venous oxygen saturation during short (filled circles full line) and prolonged (unfilled circles broken line) Mean values for 10 subjects

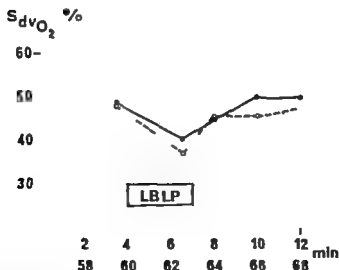


Figure 4 Effect of lower body low pressure (LBLP) on deep venous oxygen saturation during short and prolonged work Symbols as in Fig 3 Mean values for 6 subjects

Chapter 5

VASCULAR RESPONSE TO HYPEROSMOTIC STIMULATION DURING AND AFTER PROLONGED AND SHORT WORK (Paper V)

During prolonged forearm work the local hyperosmolality which has been suggested as an important mediator of muscle hyperaemia gradually decreases again as evidenced by the osmolality of the effluent venous blood and still the blood flow is maintained (Paper II). If osmolality is an important factor operating also during prolonged work the vascular response to changes in osmolality must be assumed to become more pronounced with the duration of work. To test this hypothesis the effect of an increase in blood osmolality on forearm blood flow in connection with short and prolonged work was compared. Infusions of a hyperosmotic solution was made both 1/ during and 2/ shortly after short and prolonged work.

Procedures 1 Eight subjects performed a continuous forearm work at an average load of 8 kpm/min for 68 min. A 20 per cent solution of glucose was infused through a catheter in the brachial artery at a rate of 5 ml/min for 3 min from the 4th and the 60th minute. Deep venous blood for the determination of O_2 saturation and osmolality was sampled before, at the end of and again after the infusion periods (Fig. 2 Chapter I). With the assumptions discussed in Chapter I changes in deep venous O_2 saturation were taken to indicate changes in forearm muscle blood flow.

2 Ten subjects performed forearm work at an average work load of 8 kpm/min for 5 and 55 min. Forearm blood flow was recorded plethysmographically for 12 min after each work period and 20 per cent glucose was infused at a rate of 3 ml/min for 4 min starting at 3 min after the work periods (Fig. 1 Chapter I).

Results After 3 min work deep venous osmolality had increased by about 7 mosm/kg and after 60 min work it had decreased again to a value not significantly above the resting venous level. The infusion of hyperosmotic glucose during work increased deep venous osmolality to the same extent 8 mosm/kg at 4 and 60 min. The infusion at 4 min was accompanied by a 23 per cent increase in deep venous O_2 saturation indicating an increase in forearm blood flow. After 60 min work however the infusion of glucose did not affect the O_2 saturation.

After short and prolonged work the infusion of hyperosmotic glucose was accompanied by a similar flow increase being slightly more than 100 per cent in both situations. In the individual subjects blood flow showed marked oscillations during the infusions.

Discussion It might be argued that the extent to which adequate mixing between blood and the infusate has been obtained is uncertain. According to Wahren (1968) a higher infusion rate 30 ml/min is required during light work for complete mixing.

However it does not seem likely that the degree of mixing would differ systematically between infusions performed in connection with short and prolonged work since the blood flow at the start of infusion is similar in the two situations. Furthermore in the experiments where the hyperosmotic solution is infused during work conclusions regarding changes in forearm blood flow are drawn from the O_2 saturation of the venous blood draining the working muscle. The estimation of the extent to which the infusion affects blood osmolality is made on blood from the same vein.

Thus in addition to the fact that deep venous osmolality decreases with the duration of work the results show that the sensitivity to hyperosmolality is not increased but rather reduced during prolonged work. Consequently this factor cannot be ascribed a general role as a mediator of exercise hyperaemia although it might well be of importance during the early phase of muscular work.

Chapter 5

MODIFICATION OF THE FOREARM BLOOD FLOW RESPONSE TO CONTRALATERAL ISOMETRIC HANDGRIP BY SHORT AND PROLONGED WORK (Papers IV and VI)

Exercise changes the vasomotor nervous outflow and provided this change is generalized the effect of altered vasomotor influence might be studied in a limb not taking part in the exercise where the response is not modified by local metabolic and mechanical factors. According to Lind et al (1964) the vascular resistance of the resting arm increases during contralateral isometric contraction. In some of their subjects however a decrease was found but was attributed to inadvertent muscle activation. Bevegård and Shepherd (1966) found an increase in vascular resistance in the resting arm also during dynamic leg work but this was preceded by an initial decrease in resistance.

In the present study the pattern of blood flow response in the resting arm to contralateral isometric contraction and the neurogenic mechanisms behind it were evaluated. The stimulus of contralateral handgrip was then utilized to affect blood flow in a forearm resting after prolonged and short work respectively.

Procedure In all 29 subjects were studied. Isometric contraction was performed as a handgrip at $1/3$ MVC or as a dorsiflexion of the foot at $1/2$ MVC. ECG and arterial pressure were recorded continuously. Blood flow in the resting arm was measured plethysmographically. To ascertain that inadvertent muscle activation did not influence blood flow in the resting arm EMG was recorded in some experiments. The contralateral isometric contraction was repeated after regional β - and α -adrenergic blockade produced by close intraarterial infusion of either 0.5 mg propranolol given slowly in a single dose or 200 μ g phentolamine/min as a continuous infusion. In the last series forearm blood flow was followed plethysmographically for 17 min after short and prolonged forearm work at a load of 8 kpm/min and contralateral isometric handgrip was applied at 3, 8 and 13 min. In connection with the second of these contractions deep venous blood from the resting forearm was also sampled.

Results Arterial mean pressure increased linearly during isometric contraction. Blood flow in the resting arm increased rapidly to a maximal value of two and a

half times the precontraction flow. Towards the end of contraction it decreased again but still remained twice the precontraction value, thus vascular resistance remained below that before contralateral handgrip. The increase in flow was not due to inadvertent activation, since maximal flow was recorded early during contraction when electromyographic activity was only rarely recorded. Dorsiflexion of the foot caused the same pattern of blood flow response in the resting forearm as did contralateral handgrip.

Local β -adrenergic blockade reduced the flow increase during contralateral handgrip substantially. During α -adrenergic blockade the flow increase continued throughout the contraction period and no tendency to flow decrease was seen towards the end of the contraction.

The blood flow in the previously working forearm was increased by contralateral handgrip to a greater extent after prolonged than short-term work. The comparison was rendered somewhat difficult by the fact that precontraction flow often differed after short and prolonged work. However, the difference in response remained even if pairs of contractions were compared where precontraction flow was similar.

Discussion Contralateral handgrip induced a rapid increase in the resting forearm blood flow appearing before any substantial increase in arterial pressure signifying a decrease in vascular resistance. Thus the flow increase must to a great extent be the result of neurogenic vasodilator mechanisms. Since it was considerably reduced after regional β -adrenergic blockade the nature of the mechanism is evidently β -receptor activation. The relative increase in resistance towards the end of the contralateral contraction which was abolished by α -adrenergic blockade might be interpreted as the result of a gradual change to predominantly α -adrenergic effects. The neurogenic vasomotor effects seem to be generalized since the same pattern of flow response was induced by leg and hand contraction. However, in addition to the mentioned neurogenic effects distension by the increased arterial pressure might contribute to the flow increase and vascular smooth muscle reaction to the distending force might be involved in the terminal relative flow decrease.

The more marked flow increase produced by contralateral handgrip during the hyperaemia after prolonged than after short work might be interpreted as the result

of increased β -adrenergic effects especially since the difference is most pronounced during the early phase. The extent to which such an interpretation is valid is somewhat unclear in the light of the earlier described finding of a less pronounced effect of isoproterenol on forearm blood flow after prolonged work. Thus tentatively, a preliminary interpretation would be that prolonged work also produces a diminished tendency to respond with an increase in myogenic tone to the intravascular pressure increase during isometric contraction.

Chapter 7

GENERAL DISCUSSION

Factors which might mediate the dilatation of the muscle vascular bed during exercise and adapt blood flow to the increased oxygen demand have been the subject of numerous investigations. Local chemical and physical changes caused by the increase in metabolic rate are generally believed to produce the flow increase (for review see Mellander and Johansson 1968, Ross 1971) and several substances released from the working muscle have vasodilator effects. The increased concentration of lactate, H^+ and K^+ , the increase in PCO_2 and decrease in PO_2 as well as the increased osmolality may all contribute but their relative importance is not established. The duration of work has been shown to affect the release of several of the above mentioned substances such as lactate, H^+ and CO_2 (Wahren and Hagenfeldt 1968, Jorfeldt 1970) without affecting exercise hyperaemia to a major degree and work duration can be expected to influence the local conditions in the working muscle also in other respects. Thus it seems reasonable to assume that the release also of other vasodilator factors may change with the duration of work or that the vascular response to a given vasodilator stimulus is altered.

The aim of the present study was to elucidate the relative importance of a number of locally active substances by relating the muscle blood flow to their release as reflected by their concentrations in the effluent venous blood in connection with exercise of well defined modes, intensities and durations. Special attention was given to possible changes in the relation of blood flow to the release of vasoactive substances during prolonged work and to possible changes in the response of the muscle vascular bed to vasoactive stimuli.

Exercise performed with the forearm was used since the active muscle mass is small and the amount of metabolites produced is small enough not to affect their concentration in the arterial blood. Furthermore the musculature is usually supplied by one artery, the brachial artery, permitting the infusion of substances for the study of their effects on forearm blood flow and due to the small tissue mass in amounts which do not produce systemic effects. Furthermore the deep venous system of the forearm drains almost exclusively muscle tissue thus permitting the sampling of blood for analyses of substances released from the active muscle (Coles et al 1958, Idbohrn and Wahren 1964).

The forearm consists of muscle tissue to about 60 per cent (Abramson and Ferris 1940 Cooper, Edholm and Mottram 1955) Thus under conditions of increased muscle blood flow such as postexercise hyperaemia blood flow measured plethysmographically probably to a great extent represents muscle blood flow, since skin blood flow has been shown to be unchanged by dynamic forearm work of moderate duration (Wahren 1966) Furthermore the direct effects on skin vascular resistance of changes in skin temperature, within the range observed during prolonged work in the present study are apparently small (Catchpole and Jepson 1955 Sealman 1945 Paaske Hovind and Sejrson 1973)

In studies on the blood flow response to intra-arterial infusion of different vasoactive substances the extent to which adequate mixing is accomplished must be taken into consideration According to Wahren (1968) an infusion rate of 30 ml/min is required during moderate work in order to obtain complete mixing In view of the possibility that the infusion as such then may affect the blood flow and render to response to different stimuli difficult to evaluate lower rates of infusion have been used and thus the extent to which mixing has been obtained is uncertain However conclusions are drawn from paired comparisons between effects of infusions in connection with short and prolonged work at similar levels of blood flow and hence there is no reason to believe that the degree of mixing would differ systematically

The decline in blood flow was initially somewhat faster after prolonged than after short work but levelled off after 2-3 min at 3 times the resting value whereas after short work blood flow continued to decrease gradually At 3 min the blood flow in most instances was at a similar level after the short and the prolonged work and this point of time was therefore chosen for the start of infusion of vasoactive substances or the application of LBLP However because of the difference in course of spontaneous flow decrease it was still difficult to draw firm conclusions regarding differences in response to vasoactive stimulation after short and prolonged work Consequently only substantial differences in response or responses appearing very early during the period of stimulation or in the opposite direction of the difference in spontaneous flow decrease after short and prolonged work can be taken as evidence of an altered vascular response induced by prolonged work

The concentration of vasoactive substances in effluent venous blood produced by short-term muscle activity was first studied using isometric contractions at varying fractions of MVC. Osmolality showed the closest parallelism to postexercise blood flow both with regard to maximal values and the time course for the return towards resting level which is in accordance with the contention brought forward by Mellander and co-workers (Lundvall, Mellander and White 1969; Mellander and Lundvall 1971; Lundvall 1972) and local osmolality may under these conditions play an important role. However, from the present findings it cannot be excluded that both lactate, in addition to its contribution to osmolality, pH and PCO_2 may also be considered as possible factors contributing to the postcontraction hyperaemia. Deep venous K^+ concentration and PO_2 did not follow the flow changes either with respect to magnitude or time course and thus it seems reasonable to conclude that they do not contribute significantly to the hyperaemia after the isometric contraction.

In accordance with the findings by Wahren and Hagenfeldt (1968) and Jorfeldt (1970) blood flow immediately after prolonged dynamic forearm work was the same or slightly higher than after short work. In addition to a decrease in deep venous lactate, CO_2 and H^+ during prolonged work that has been demonstrated earlier (Bang 1936; Wahren and Hagenfeldt 1968; Jorfeldt 1970), osmolality and K^+ concentration which were significantly raised during short work did not differ from resting levels at the end of prolonged work. It can therefore be concluded that during prolonged work muscle blood flow could not have been maintained by these factors unless either the response of the vascular bed to them was increased or the neurogenic or intrinsic myogenic vasomotor effects on vascular resistance had changed.

Of these alternatives the possibility of an altered response to factors related to the increased metabolism was first taken into consideration. Since Mellander and co-workers (Mellander et al. 1967; Lundvall, Mellander and White 1969; Jonsson 1970; Lundvall 1972) on the basis of intensive studies on the vasodilating effect of hyperosmolality put forward the hypothesis of osmolality as an essential physiological regulator of regional blood flow, it seemed important to study the effect of increased osmolality on forearm blood flow during exercise of varying duration. An increase in local osmolality elicited by the infusion of a hyperosmotic glucose solution did raise deep venous oxygen saturation indicating an increase in blood flow during short but not prolonged work. This finding implies a diminished response

of the muscle vascular bed to hyperosmotic stimulation during prolonged work while at the same time the local hyperosmolality induced by the muscle activity is less pronounced. Consequently it appears reasonable to conclude that while local hyperosmolality may be an important factor contributing to the increase in muscle blood flow during work of short duration it cannot be of significance in the regulation of blood flow during prolonged work.

Since the results did not support the conception that the response to the vasodilator action of hyperosmolality increases with prolonged work the possibility that prolonged work alters the response to adrenergic stimulation was also tested.

The response to increased sympathetic nerve activity induced by lower body low pressure was probably not different in connection with prolonged compared to short work. However, the difference in response after short and prolonged work is somewhat difficult to interpret because of the difference in spontaneous rate of flow decrease. On the other hand when noradrenaline was infused intra-arterially during work a more marked flow reduction was found in connection with prolonged than with short work. After prolonged work a qualitative change in response was observed to the effect that the infusion of noradrenaline was followed by a substantial increase in flow which was not seen after the short work and which could be abolished by β -adrenergic blockade.

It is conceivable that the effect of circulating noradrenaline on the vascular smooth muscle is changed by prolonged work increasing both the α - and the β -adrenergic stimulating properties. The qualitatively altered response might be of importance for the maintenance of blood flow during prolonged work although it can certainly not fully explain the unaltered exercise hyperemia.

These findings prompted a further study of the effect of β -adrenergic stimulation and of α -adrenergic blockade on the hyperemia after prolonged and short work respectively. The infusion of isoproterenol could not confirm an increased response to β -adrenergic stimulation at least not to the type of stimulation used. The response to phentolamine might indicate an increased adrenergic vasoconstrictor stimulation or an increased sensitivity to it after prolonged work but earlier studies seem to give no support to such an interpretation. Since other effects of the drug have been described (Taylor et al 1965 a, b and c) an additional explanation seems to be that prolonged work

Induces a more pronounced response to its direct vasodilating effects

Finally the response of the muscle vascular bed to vasoactive stimulation in connection with prolonged and short work was studied also as the response to a contralateral isometric handgrip

An isometric contraction performed as a handgrip could be shown to increase blood flow in the contralateral resting forearm on an average 2.5 times the resting flow indicating a decreased vascular resistance. A further analysis suggested that β -adrenergic effects are partly responsible for this flow increase especially during the early phase of contraction while the continuously rising arterial pressure might become more important towards the end of the contraction period when also a probable increase in sympathetic vasomotor activity might become more vasoconstrictive in effect as indicated by the relative increase in resistance towards the end of the contraction

Contralateral handgrip contraction increased blood flow in the previously working forearm significantly more when performed during the postexercise hyperaemia after prolonged than after short work. Since the flow increase produced by contralateral handgrip apparently is the result of a complex interaction between neurogenic sympathetic effects, arterial pressure increase and a myogenic reaction of the vascular smooth muscle induced by the increase in intravascular pressure the implication of a larger and more rapid flow increase after prolonged work is not obvious

The far more rapid onset of the flow increase after prolonged work suggests that after all a higher sensitivity to β -adrenergic stimulation is induced but since the earlier studies gave little support to the contention of an increased sensitivity to β -adrenergic stimulation a preliminary interpretation of the results may be that prolonged work in addition lowers the reactivity of the vascular smooth muscle to the increase in arterial pressure. A lowered intrinsic myogenic tone might then be a factor contributing to the maintenance of flow during prolonged work

CONCLUSIONS

1 The local increase in osmolality might contribute significantly to the blood flow increase in exercising muscle during work of short duration

2 During prolonged work osmolality decreases towards the resting level and in addition the response of the vascular bed to an increase in blood osmolality is reduced. Consequently hyperosmolality cannot be ascribed a general role as mediator of exercise hyperaemia

3 Prolonged work induces quantitative and qualitative changes in response to adrenergic stimulation but not of a magnitude that explains the maintenance of blood flow

4 The considerable flow increase induced by phentolamine which was found after prolonged work is therefore to a large extent interpreted as a more marked response to a nonadrenergic smooth muscle relaxing property of the compound and thus the vascular response to non-neurogenic stimuli seems to be altered by prolonged work

5 A strong isometric contraction induces an increase in blood flow in the resting arm to a great extent due to β -adrenergic effect during the initial phase. The pattern and magnitude of the response is not dependent on the muscle group used indicating a generalized increase in adrenergic vasomotor activity during contraction

6 Contralateral isometric contraction performed after short and prolonged work increases the blood flow in the resting arm more markedly when in its hyperaemic phase after prolonged than after short work. As differences in adrenergic reactivity between the two situations seem moderate the finding might imply a change in myogenic reactivity of the vascular smooth muscle contributing to the maintenance of blood flow in exercising muscle during prolonged work

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VASCULAR RESPONSE TO HYPEROSMOTIC STIMULATION DURING AND AFTER PROLONGED AND SHORT WORK

The increase in blood flow through exercising muscle has been ascribed to the action of several metabolically linked vasoactive substances (Mellander and Johansson 1968 Haddy and Scott 1968 Ross 1971) but the relative importance of the different factors has not been assessed. A mechanism by which several substances might act together to produce vasodilatation was offered by Mellander and co-workers (Mellander et al 1967 Lundvall Mellander and White 1969 Mellander and Lundvall 1971 Lundvall 1972) who suggested that the local hyperosmolality produced by the formation of osmotically active metabolites during exercise plays an important role in the regulation of muscle blood flow during exercise.

However as shown earlier (Kalljer and Eklund 1974) the osmolality of the effluent venous blood that increases during the early phase of muscular work is significantly reduced during prolonged work. At the same time blood flow is unchanged or slightly increased (Wahren and Hagenfeldt 1968 Jorfeldt 1970). If osmolality is an important general factor in the regulation of the local blood flow in exercising muscle operating also during prolonged work then the vascular sensitivity to changes in osmolality must be assumed to have increased with the duration of work.

To test this possibility the effect on forearm blood flow of increased osmolality accomplished by close intraarterial infusion of a hypertonic glucose solution was studied during and after short and prolonged forearm work.

1 Effect of close intraarterial infusion of hypertonic glucose on forearm blood flow during short and prolonged work

Subjects Eight subjects aged 25-36 years were studied

Procedure Percutaneous catheters were introduced into the brachial artery and a deep vein of the working arm. The subjects performed dynamic forearm work at a load of 8 kpm/min (Kajiser 1970). A 20 per cent solution of glucose was infused into the brachial artery at a rate of 5 ml/min for 3 minutes from the fourth and the sixteenth minute of a continuous work period of 68 min duration. Arterial blood for the determination of osmolality was sampled at rest and before the infusion periods. Deep venous blood for the determination of osmolality and oxygen saturation was sampled at rest and in duplicate before and again at the end of the infusion periods. Single samples were withdrawn at 1, 3 and 5 minutes after the infusion periods. Based on the assumption that the oxygen consumption remained constant during a continuous work at a fixed intensity and that it was not to a significant degree influenced by glucose infusion changes in deep venous oxygen saturation were taken as evidence of changes in forearm muscle blood flow.

Results Arterial osmolality was the same at rest and before the infusions at 4 and 60 min of the work period. At rest deep vein osmolality did not differ from that of arterial blood. After 3 min work the venous osmolality was significantly increased above the arterial and the resting venous level by about 7 mosm/kg ($p < 0.01$). After 60 min work it had decreased again to a value not significantly above the arterial and the resting venous level.

The infusion of hypertonic glucose increased deep vein osmolality to the same extent about 8 mosm/kg at 4 min and 60 min of the work period (Fig. 1).

At rest the deep vein O_2 saturation was 51.9 ± 3.8 per cent. After 3 min work it was 32.6 ± 2.0 per cent and the increase in osmolality produced by the infusion of 20 per cent glucose was accompanied by a 23 per cent increase ($p < 0.01$) in O_2 saturation to a mean value of 38.0 ± 1.3 per cent (Fig. 1). Indicating an increase in forearm blood flow. Three minutes after the infusion it had returned to the preinfusion level. After 60 min work however the infusion of glucose producing the same increase in osmolality did not affect the oxygen saturation of the effluent venous blood.

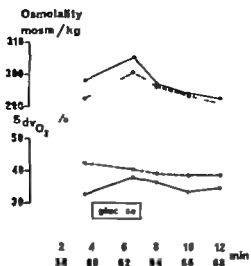


Figure 1 Effect of Intraarterial Infusion of a 20 per cent glucose solution on deep venous osmolality and oxygen saturation during short (●-●) and prolonged (○-○) work. Mean values for 8 subjects.

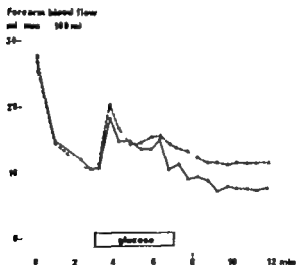


Figure 2 Effect of Intraarterial Infusion of a 20 per cent glucose solution on forearm blood flow after 5 (●-●) and 55 (○-○) min work. Mean values for 10 subjects.

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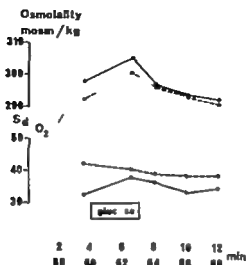


Figure 1 Effect of intraarterial infusion of a 20 per cent glucose solution on deep venous osmolality and oxygen saturation during short (●-●) and prolonged (○-○) work. Mean values for 8 subjects.

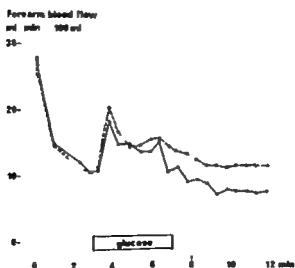


Figure 2 Effect of intraarterial infusion of a 20 per cent glucose solution on forearm blood flow after 5 (●-●) and 55 (○-○) min work. Mean values for 10 subjects.

MODIFICATION OF THE FOREARM BLOOD FLOW RESPONSE TO CONTRALATERAL ISOMETRIC HANDGRIP BY PROLONGED AND SHORT WORK

Exercise affects the vasomotor nervous outflow and the effect of the thereby altered vasomotor influence can be studied in a limb not taking part in the exercise where the response is not modified by local metabolic and mechanical factors. Lind et al (1964) studying the cardiovascular effects of an isometric contraction found that the blood flow in the resting forearm usually remained unchanged in spite of a pronounced increase in arterial pressure thus indicating an increased forearm vascular resistance. In some of their subjects however an increase in the resting forearm blood flow was recorded but this was suggested to be the consequence of inadvertent muscle activation. Eklund, Kaljser and Knutsson (1974) however found a substantial decrease in the vascular resistance of the resting forearm during contralateral isometric handgrip appearing rapidly during the contraction. Studies with EMG recordings showed that it could not be due to inadvertent muscle activation. The fact that blood flow increased rapidly at a point of time when arterial pressure was only moderately increased suggested the contribution of neurogenic mechanisms apart from a passive distention produced by the increased intravascular pressure. The aim of the present series of investigations was to study how the flow response to contralateral isometric handgrip is modified by a previous prolonged and short forearm work since earlier studies indicated a changed reactivity of the vascular bed after prolonged work (Kaljser and Eklund 1974, Eklund and Kaljser 1974). To facilitate an evaluation regarding the extent to which adrenergic neurogenic mechanisms contribute to the blood flow response the contralateral handgrip was also performed with the resting forearm under the influence of selective α - and β -adrenergic blockade.

1 Effect of regional β - and α -adrenergic blockade on the forearm blood flow response to contralateral isometric handgrip

Subjects Six subjects aged 22-42 years were studied

Procedure For the infusion of drugs and for pressure recording a catheter was introduced percutaneously into the brachial artery of the resting arm. Isometric contraction with the contralateral arm was performed as a handgrip with 1/3 of maximal voluntary capacity (MVC) for 2 minutes. MVC ranged from 45-60 kp. Blood flow in the resting forearm was recorded plethysmographically for 2 minutes before contraction, during contraction and for 2 minutes after contraction. ECG and arterial pressure were recorded continuously.

The subjects performed three contractions with blood flow recording in the resting arm without adrenergic blockade, three contractions after regional β -adrenergic blockade of the resting arm and three contractions after regional α -adrenergic blockade. To obtain β -adrenergic blockade 0.5 mg propranolol was infused slowly during 1 minute through the arterial catheter in the resting arm 4 minutes before the start of each contraction (Johnsson 1967). α -adrenergic blockade was produced by the continuous infusion of phentolamine through the arterial catheter in a dose of 200 μ g/ml and minute, starting 4 minutes before each contraction. Arterial mean pressure was recorded simultaneously.

Results In the control situation the flow increase in the resting arm during contralateral isometric contraction was of the same order of magnitude as in the previous study (Eklund, Kaijser and Knutsson 1974) and so were the changes in heart rate and arterial pressure. Heart rate and arterial pressure changes were not significantly affected by the drugs given.

After regional β -adrenergic blockade the forearm blood flow was 3.8 ± 0.4 ml/min $\times 100$ ml, which is not significantly different from the control situation. The flow increase produced by contralateral isometric handgrip was markedly less pronounced than without blockade ($p < 0.05$), especially during the early part of the contraction (Fig. 1).

α -adrenergic blockade increased forearm blood flow before contraction to 7.6 ± 1.3 ml/min $\times 100$ ml. The magnitude of increase in flow in the resting forearm during

contralateral handgrip was not significantly different from that in the control situation. However, the forearm blood flow increased continuously towards the termination of contralateral handgrip with no tendency to a terminal flow reduction (Fig. 1).

Discussion β -adrenergic blockade by propranolol did not significantly alter the basal forearm blood flow but reduced the flow increase caused by isometric contralateral handgrip, especially during the first minute of contraction. The findings thus support the conception that β -adrenergic receptor stimulation is important for the flow increase. If the assumption is made that β -receptors were completely blocked, about 50 per cent of the flow increase is due to β -adrenergic effects.

During α -adrenergic blockade by phentolamine the basal flow was doubled. This makes a direct comparison with the flow response in the control situation difficult. However, the flow increase in the resting arm continued throughout the contraction period. This finding may indicate that the relative increase in resistance towards the end of contraction, seen in the control situation, could have been the result of a gradual change to predominantly α -adrenergic effects, presumably as a result of an increased sympathetic nerve activity which in turn probably was induced by the increased effort to hold the contraction (Clarke et al. 1957; Freyschuss 1970).

2 Effect of short and prolonged work on the forearm blood flow response to contralateral isometric handgrip

Subjects Twelve subjects aged 20-36 years were studied

Procedure For arterial pressure recording a brachial artery was catheterized percutaneously. For the determination of oxygen saturation a catheter was also introduced into a deep vein of that forearm which was to perform the short and the prolonged dynamic work.

Forearm blood flow was recorded plethysmographically. Before the start of dynamic forearm work two recordings of the blood flow reaction in the resting arm during contralateral isometric handgrip were undertaken. The subject then performed dynamic forearm work (Kajiser 1970) at a load of 8 kpm/min for 5 minutes. Forearm blood flow recording was started as soon as possible after work and the flow recording was continued for 17 minutes. During this period contralateral isometric handgrip was performed three times namely at 3, 8 and 13 minutes after work and held for 2 minutes each time. The subject then worked for 55 minutes and the prolonged work was followed by flow recording and contralateral handgrip as after the short work.

Arterial blood pressure and ECG were recorded continuously. Deep venous blood for the determination of oxygen saturation was sampled in connection with the second contralateral isometric handgrip before dynamic forearm work and after the 5 and 55 min work periods. Sampling was performed immediately prior to and at 30, 60, 90 and 120 seconds of contralateral handgrip and again at 30 and 90 seconds after it. Deep venous oxygen saturation was determined also at the end of the 5 and 55 min dynamic work.

Results The effect of a contralateral handgrip on the blood flow in the resting forearm did not differ from that earlier recorded (Eklund, Kajiser and Knutsson 1974) nor did the effect on heart rate and arterial mean pressure (Fig. 2). Deep venous oxygen saturation being 53.0 ± 3.8 per cent before contraction increased to a maximal value of 65.9 ± 3.3 per cent during the contralateral handgrip ($p < 0.001$) (Fig. 2).

Heart rate and arterial pressure changes in the individual subjects were well reproducible when handgrip was repeated after short and prolonged work. The blood flow response to contralateral isometric handgrip during the hyperaemic phase following short and prolonged work

is shown in Fig 3. The absolute increase in forearm blood flow was significantly larger after the prolonged than the short work ($p < 0.001$ for paired comparison of all contractions at corresponding times after work). The difference is most evident during the first 45 seconds of contralateral handgrip.

Deep venous oxygen saturation was 36.5 ± 2.3 per cent at the end of the short work. At the end of the prolonged work it was slightly higher, being 42.9 ± 3.0 per cent ($p < 0.01$). During the second contralateral isometric contraction performed after the short and the prolonged work, it increased significantly as shown in Fig 4 ($p < 0.001$ in both situations).

As earlier described (Kajiser and Eklund 1974) the blood flow tended to be greater after the prolonged than the short work and hence the percentual increases did not differ significantly. To facilitate comparison of the response after short and prolonged work, those pairs of contractions were analyzed separately where forearm blood flow did not differ more than 25 per cent before the start of the contralateral handgrips after short and prolonged work. Twelve pairs fulfilled this criterium. Of these six were from the first handgrip after the exercise periods, four from the second and two from the third (Fig 5). In these cases both the relative and the absolute increase in blood flow were significantly greater after the prolonged work ($p < 0.05$ and < 0.001 respectively).

In order to examine if the magnitude of the blood flow response to contralateral handgrip was dependent upon the precontraction flow level, the maximal absolute as well as the maximal relative flow increase during contraction was plotted against the mean flow during the 30 seconds immediately prior to contraction (Figs 6 and 7). Data are presented for all contractions: those performed at rest as well as during the hyperaemic phase after the short and the prolonged work. The absolute increase was not dependent upon the precontraction blood flow level.

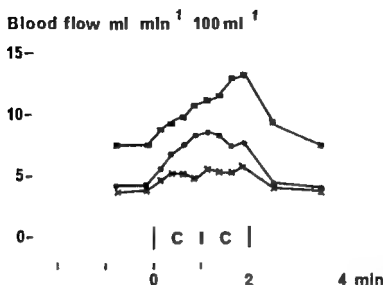
Discussion The fact that the forearm blood flow remains at a higher level after prolonged work renders a comparison of the blood flow response to contralateral isometric handgrip after short and prolonged work difficult. However, the increase in forearm blood flow seems not to be dependent upon the blood flow level before contralateral contraction. During the first contralateral handgrip in the hyperaemic phase after the short work

the blood flow increase is superimposed on the spontaneous flow decrease (Kajiser and Eklund 1974) and thus the flow increase produced by contralateral contraction will be somewhat underestimated. After the prolonged work however, the flow increase is significantly greater already after 45 seconds of the contraction when this source of error should be of little importance. Thus it seems reasonable to conclude that there is a difference in blood flow reaction of the resting arm to contralateral isometric handgrip induced by a preceding prolonged dynamic work. This conclusion is further supported by the finding that the increase in deep venous saturation is the same after the short and the prolonged work. Assuming a constant arterial oxygen saturation and no difference in oxygen consumption of the forearm muscles at the second contralateral handgrip, the calculated a-v O_2 difference shows a larger percentage decrease after the prolonged work. Furthermore, the increase in deep venous oxygen saturation supports the contention that the flow increase to a great extent occurs in the forearm muscles.

The blood flow increase in the resting forearm during contralateral isometric contraction is probably the result of a complex interaction between mechanical distension (Folkow and Löfving 1956), changes in the intrinsic myogenic tone as a response to the increase in pressure (Bayliss 1902, Folkow 1949) and sympathetic neurogenic effects as earlier discussed (Eklund, Kajiser and Knutsson 1974). In the present study it has been shown that of these factors β -adrenergic effects were of importance, especially during the early phase of contralateral contraction. However, as earlier experiments (Eklund and Kajiser 1974) have demonstrated, prolonged work does not increase the sensitivity to β -adrenergic stimulation, i.e. the response to isoproterenol was less pronounced after prolonged work. The more marked flow increase in the resting arm produced by contralateral handgrip in the hyperaemic phase after prolonged work might then tentatively be interpreted to a substantial degree to result from a reduced tendency to respond with an increase in myogenic tone to intravascular pressure increase. This altered mode of response might contribute to maintain flow during prolonged work.

Conclusion A contralateral isometric handgrip that has been shown to increase the resting forearm blood flow does so also in a resting forearm in the hyperaemic phase after work. The induced flow increase is more pronounced

after a prolonged than after a short work β -adrenergic effects contribute to the flow increase. However, since earlier studies have not shown any major change in β -adrenergic sensitivity after prolonged work, the more pronounced flow response to contralateral handgrip after prolonged work is probably to a great extent due to a reduced myogenic response.



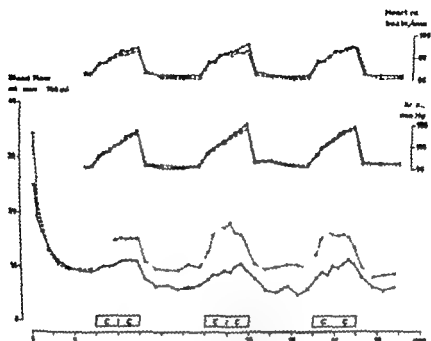


Figure 3 Effect of contralateral isometric contraction on heart rate and arterial pressure and on forearm blood flow after 5 min (● ●) and 55 min (○ ○) forearm work. Mean values for 12 subjects.

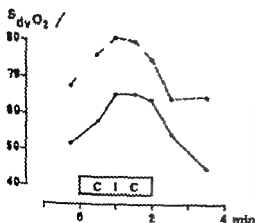


Figure 4 Effect of the second contralateral isometric contraction on deep venous oxygen saturation during the hyperaemia after 5 (● ●) and 55 min (○ ○) forearm work. Mean values for 12 subjects.

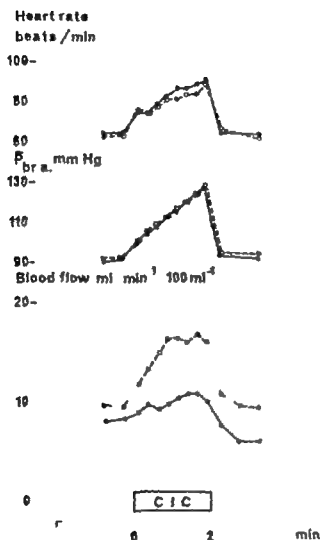


Figure 5 Effect of contralateral isometric contraction on heart rate and arterial pressure and forearm blood flow after short (●-●) and prolonged (○-○) work. Pairs of contractions where forearm blood flow before the start of the contralateral isometric contraction did not differ more than 25 per cent. Mean values for 12 pairs.

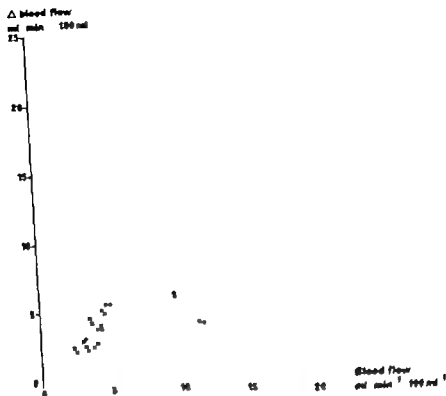


Figure 6 Maximal absolute flow increase during contralateral isometric contraction in relation to precontraction flow. Values for contractions performed at rest (x) after 5 min work (●) and after 55 min work (○)

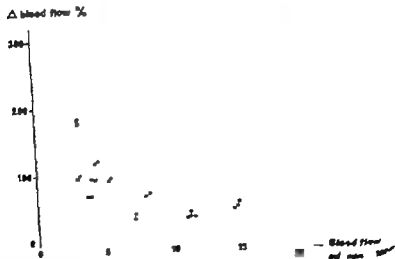


Figure 7 Maximal percentage flow increase during contralateral isometric contraction in relation to precontraction flow. Symbols as in Fig. 6

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 402

THE ORGANIZATION OF THE ASCENDING
CATECHOLAMINE NEURON SYSTEM
IN THE RAT BRAIN
AS REVEALED BY THE GLYOXYLIC ACID
FLUORESCENCE METHOD

BY

OLLE LINDBALL and ANDERS BJÖRKLUND

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FROM THE DEPARTMENT OF HISTOLOGY
UNIVERSITY OF LUND LUND SWEDEN

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INTRODUCTION

The noradrenaline (NA)- and dopamine (DA)-containing cell groups in the lower brain stem are known to give rise to widespread ascending projections to many diencephalic and telencephalic regions. They can be regarded as highly interesting components of the ascending pathways from the brain stem reticular formation. Although much information has been gained about the projections of these catecholamine (CA) neuron systems, above all through the extensive work of Fuxe, Ungerstedt and collaborators ^{1, 19, 21, 34, 35, 37} and of Maeda and collaborators ^{41, 42} the knowledge of the organization of the CA systems is still far from complete. This can at least partly be referred to the difficulties inherent in the methodology employed, which usually requires the use of lesions of the axons in order to increase the CA content of their non terminal parts to levels sufficient for their visualization by the Falck Hillarp formaldehyde fluorescence method.

The introduction of the glyoxylic acid (GA) fluorescence method ^{7, 25, 26} provided a new and very useful tool for sensitive and detailed morphological studies of central NA and DA neurons. From our preliminary observations with this technique in the rat brain it became evident that the GA method reveals a considerably richer and much more informative picture of the CA fibre systems than was previously possible and that it allows the direct tracing — in the untreated intact animal — of the CA axons from their cell bodies of origin up to their ultimate ramifications. Furthermore several so far unknown CA-containing axon systems were detected in the GA treated specimens. In the present study the GA method was employed for a description of the principal organization of the ascending CA pathways from the brain stem in the rat. This account is primarily concerned with the identity, composition, course, and primary branching patterns of the rostrally projecting CA fibre tracts, and attempts have been made to relate these systems to established neuroanatomical nomenclature.

MATERIAL AND METHODS

Brain tissue was studied from about 300 female Sprague-Dawley rats (180—250 g body weight). The *fluorescence histochemical analysis* was performed with the GA method according to Lindvall and Björklund ²⁵. Briefly the procedure was: brain pieces from GA-perfused rats were sectioned at about 30 μ (frontal sagittal and horizontal planes) in a Vibratome Instrument (Oxford Instruments, San Mateo Calif., USA). The sections were immersed in a GA solution, dried, and treated with GA vapour or heated at +100 C according to the procedure described by Lindvall and Björklund ²⁵. As controls for the specificity of the studied fluorescence non-GA treated sections from fresh non-perfused brains were used.

The *microspectrofluorometric analysis* was performed according to Björklund, Ehinger and Falck ⁴ and Björklund, Falck and Owman ⁵ with a Leitz microspectrofluorometer. The spectra thus obtained were compared with those of the

GA-induced fluorophores of authentic CAs in histochemical models and in tissues²⁵

The nomenclature used follows essentially that of König and Klippel²² and Zeman and Innes²⁶ these sources were also used in the preparation of some of the drawings.

RESULTS

In the GA method the intraneuronal primary CAs, NA and DA yield a UV stable, blue or greenish-blue fluorescence (depending on the filter setting), making fluorescent the entire axons, the cell bodies, and variable portions of the dendrites of the CA-containing neurons. However the morphology of the perikarya is often more or less distorted. The identity of the studied structures as CA-containing was established according to the specificity criteria given by Lindvall and Björklund²⁵ Randomly selected structures in various parts of the brain, having the characteristic greenish-blue GA-induced fluorescence were analysed microspectrofluorometrically and the spectral characteristics were found to be identical to those of the GA-induced fluorophores of DA and NA (cf ref 35). The secondary CA, adrenaline, yields very low fluorescence in the GA method²⁵ possible adrenaline-containing structures can therefore be expected not to show up in the GA-treated sections. With the GA-perfusion and immersion procedure employed in the present study indolamine (e.g. serotonin)-containing structures showed a very weak and variable fluorescence also after MAO-inhibition. Their fluorescence was brownish-yellow and faded fairly rapidly upon UV irradiation thus being easily distinguishable from the fluorescence of the CA-containing structures.

The ascending CA fibre systems originating in mesencephalic, pontine, and medullary DA- and NA-containing cell groups and projecting through the lower brain stem towards diencephalic and telencephalic regions were traced in serial sagittal, frontal, and horizontal sections. The following description refers to these ascending fibre pathways. Thus, branching patterns and local projections within the lower brain stem — as well as projections towards cerebellum and the spinal cord — are dealt with only to a very limited extent.

The ascending CA axons originating in pontine and medullary cell groups were confined to two major fibre systems. One system ascends along the tegmental fascicles of Forel²⁰ It is partly loosely arranged and occupies almost the entire cross-section of the pontine and mesencephalic tegmentum, in a position corresponding to the *central tegmental tract (CTT)* as defined by e.g. Bürgi and Bucher²² Nauta and Kuypers²³ and Wüschler *et al.*²⁷ with classical neuro-anatomical techniques (cf Discussion). In the dorsomedial part of the pontine and mesencephalic CTT the CA fibres are more densely aggregated and typically arranged in fascicles. These aggregated fascicles are distinguished as a separate bundle within the CTT it is identical with the dorsal CA bundle of Ungerstedt²⁴ In the following it is referred to as the *dorsal segmental bundle (DTB)*

The second major fibre system is a prominent periventricular-periaqueductal

emergence from the locus coeruleus however the further course of these crossing fibres was not determined.

Along its course through mesencephalon the DTB gives off fibres in several directions. Within and just rostral to the decussation of the superior cerebellar peduncles, fibres leave the DTB rostroventrally (and somewhat laterally) along the so-called tegmental radiations, which will be described in more detail below. These fibres were seen to consist both of axons deviating from the bundle, and of collateral branches from axons continuing rostrally. Laterally many single fibres leave the DTB (both as deviating fibres and as collaterals) towards the dorsal tegmental regions and the geniculate bodies. Dorsally two branches of the DTB project into the posterior and anterior colliculi, respectively. The branch to the posterior colliculus — consisting primarily of collateral fibres — leaves the DTB in a dorsal and slightly caudal direction at the level of the decussation of the superior cerebellar peduncles. The branch to the anterior colliculus deviates from the DTB somewhat further rostrally in a rostradorsal direction. This branch consists of fascicles of DTB fibres deviating from the lateral part of the main bundle.

At the meso-diencephalic junction the DTB is situated ventrolateral to the periventricular gray of the third ventricle, close to Darkschewitsch's and Cajal's nuclei. From here, the main bundle bends ventrally and somewhat laterally to pass in between the fasciculus retroflexus and the medial lemniscus, and then just lateral to the mamillothalamic tract. Here CA cells belonging to the caudal thalamic A11 group were observed to project axons into the DTB. Gradually along its course through the middle hypothalamus, the DTB merges with the MFB system. As described in more detail by Lindvall *et al.*²⁷ the DTB gives off several branches in the caudal diencephalon that contribute importantly to the adrenergic innervation of the thalamus (see Fig. 1). Thus, ventral to the posterior commissure fibres leave the bundle dorsally to cross the midline in the commissure. Some of these fibres run out into the contralateral pretectal region and others probably join the contralateral DTB. When passing between the fasciculus retroflexus and the medial lemniscus, one component leaves the bundle dorso-rostrally along the surface of the fasciculus retroflexus and another bends laterally to fan out on the dorsal surface of the medial lemniscus. These branches of the DTB project into the medial and midline regions and the ventrobasal complex, respectively of the thalamus. Further rostrally a fourth branch is seen to deviate from the DTB along (within and along the surface of) the mamillothalamic tract to reach the anterior thalamic nuclei. At the level of the subthalamic nucleus — shortly before the bundle joins the MFB — some DTB fibres bend laterally and somewhat dorsally to run intermingled with fibres of the nigrostriatal DA system (see below) into the internal capsule. Here some of them were seen to take a rostroventral course towards the CA fibre systems in the supranuclear decussations and the ansa lenticularis (dealt with in more detail below). Others could possibly reach the cortex through the neostriatum. The further rostral course of the DTB within the MFB system is described below in connexion with the MFB.

B THE CENTRAL TEGMENTAL TRACT

The fibres of this CA system could, in sagittal sections, be traced along the surfaces of the myelinated tegmental fascicles from the medulla oblongata into the internal capsule and the supraoptic decussations (see Fig. 2). The tract is richest in CA axons, and has its maximum extension, in pons and the caudal mesencephalon, here the adrenergic fibres occupy practically the entire cross section of the pontine and mesencephalic tegmentum (Figs. 2 and 6). Part of this CTT system could be followed caudally into the medulla oblongata. Here most of the CTT fibres are aggregated into a dense bundle situated just ventrolateral to the hypoglossal nucleus and ventral to the dorsal vagal nucleus. Going rostrally this bundle passes between the root fibres of the facial nerve and just ventral to the genu of the facial nerve (Fig. 3 A) and when reaching the level of the locus coeruleus the bundle deviates slightly ventrally into a position in the pontine and mesencephalic CTT immediately ventrolateral to the DTB (Fig. 2). Ventral and medial to this dense medullary CTT bundle there occurs, in medulla oblongata and pons, a system of loosely arranged longitudinally running CA fibres, many of which have a peculiar smooth (nonvaricose) appearance. These fibres are probably packed two or more close together into single fibre aggregates (see Fig. 3 B). In the sagittal sections they could be followed from the region of the caudal half of the inferior olive to the rostral pons, here they may turn medially to run further rostrally along the raphe. The origins and terminations of these fibres were not determined.

The medullary CTT bundle (MB in Figs. 1 and 2) consists of both ascending and descending fibres. A major descending component comprised axons that could be traced back to the caudal part of the principal locus coeruleus. They run first ventrally and then bend sharply caudally through and around the ventral part of the genu of the facial nerve caudally they were traced to the level of the nucleus of the solitary tract. To judge from the observations of Lolzou²⁰ after lesions in the locus coeruleus, this descending locus bundle might partly give rise to the extensive CA terminal systems present in this nucleus and in the adjoining dorsal vagal nucleus and the commissural nucleus.

The ascending medullary CA axons originate in the CA cell group in the lateral reticular nucleus (group A 1 according to the nomenclature of Dahlström and Fuxe^{1,2}) and probably also in the cell group (A 2) situated mainly within the dorsal vagal nucleus and the commissural nucleus. The axons from the cell bodies in the lateral reticular nucleus reach the CTT bundle along a straight dorsomedial course. The A 2 cells situated dorsomedially and rather close to the bundle project axons ventrolaterally into the bundle. Many of the A 1 and A 2 axons were seen in sagittal sections to branch in a T-shaped manner in the bundle to give rise to an ascending and a descending collateral.

In the pons the number of CA axons and the size of the CA fibre tract increase considerably owing to the inflow of pontine fibre systems into the CTT. In the region of the pontine CA cell groups, the CA fibre arrangements are highly complex, as several abundant axonal systems partly intermingle with

each other and with areas of dense fluorescent terminal networks. In this intricate situation the elucidation of the branching patterns, terminations, and decussations of the fibre systems within the pons must await further experimental work. However the *principal* features of the ascending systems were revealed already in the GA treated material of the intact rat.

In sagittal and frontal sections, three major inflows to the CTT were observed from the pontine cell groups. The most caudal inflow is from the CA cell group (A 5) situated lateral to the superior olivary nucleus, close to the outgoing fibres of the facial nerve. These axons run along the root of the facial nerve and join the ascending medullary CTT bundle. The second, massive inflow of fibres was from the region of the subcoeruleus CA cell bodies (A 7) along the ventral and medial aspects of the superior cerebellar peduncle (Fig. 2). It seems quite probable that also the more dorsally situated CA cells belonging to the A 4 group could contribute to this inflow. As illustrated in Fig. 2 the subcoeruleus fibres run in their initial course in a rostroventral direction and soon turn rostrally to pass through and ventral to the decussation of the superior cerebellar peduncles as a broad loosely arranged system in the CTT. Here, they join fibres coming from the laterally situated subcoeruleus cell bodies, and possibly also fibres coming from the contralateral side. In a cross section at this level (Fig. 6) the subcoeruleus fibres fill practically the entire tegmentum ventrolateral to the DTB. The fibres running in the dorsomedial part of this subcoeruleus fibre system intermingle with the medullary CTT bundle (described above) which runs through the decussation of the superior cerebellar peduncles in a position ventrolateral to the DTB (Fig. 2).

The third inflow of fibres to the CTT was from the principal locus coeruleus. These axons were of the same morphological type as those ascending in the DTB and in fact the projection of axons from the locus coeruleus into the CTT appeared in the sagittal sections as a more loosely arranged ventral extension of the DTB as illustrated in Fig. 1. The locus fibres ascending in the CTT are thus primarily located in the region ventral to the DTB and medial to the medullary and pontine bundles, and they were seen to run straight through the tegmental radiations, along a course parallel to the DTB into the MFB system.

In sagittal and frontal sections large and almost vertically oriented flows of fibres were observed (Fig. 6) interconnecting the CTT with the periventricular gray of the fourth ventricle including the locus coeruleus region. Many of these fibres are attached to the CTT fibres in a T-shaped bifurcation. Their direction is uncertain but it seems most probable (cf. refs. 16 and 64) that these vertically arranged fibre systems largely represent collateral branches from the CTT.

Fig. 2. Semidiagrammatic representation of the ascending and descending CA fibre systems in the central tegmental tract (CTT) and its caudal extension, the medullary CA bundle (MFB). Rostrally part of the nigrostriatal pathway is represented in its extension through the internal capsule (CI) and the globus pallidus. Composite drawing of different sagittal planes. Note in particular that the mesencephalic and pontine parts of the drawing illustrate a plane situated about 0.5 mm more medial than the rest. For abbreviations, see Index.

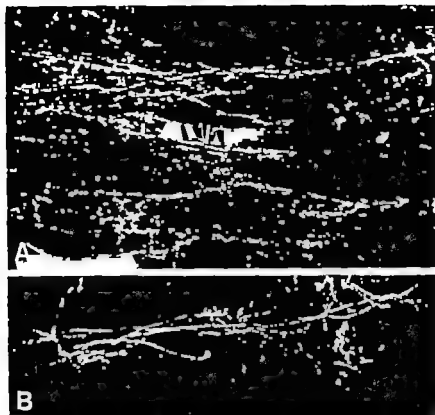


Fig 3 A. Ascending and descending fibres in the medullary bundle of the central tegmental tract at the level of the genu of the facial nerve. (X 320) B. CA fibres of the smooth, nonvaricose appearance in the rostral medulla oblongata, running medially in the central tegmental tract system. (X 240).

running dorsally to give rise to the extensive terminal systems distributed in the locus coeruleus region and in the rostral part of the dorsal raphe nucleus and the adjoining ventrolateral central gray (see below) it is also conceivable that they could contribute axons to the dorsal periventricular system.

The pontine and medullary fibres in the CTT run through and ventral to the decussation of the superior cerebellar peduncles, and on its rostral side the dorsal parts of the system turn rostroventrally to fan out along the so-called tegmental radiations (described below) (Figs. 2 and 7). As illustrated schematically in Fig. 16 these CTT fibres follow the radial course of the tegmental radiations for varying distances and a significant portion of the system leaves the radiations after only a short distance to resume their longitudinal course, now in more ventral and lateral positions. Other CTT fibres follow the tegmental radiations down into the ventrolateral tegmentum. Here they join the most ventral CTT fibres (passing ventral to the decussation of the superior cerebellar peduncles) to contribute to the formation of the MFB system (see below). Thus, rostral to the tegmental radiations, the CTT is markedly thinned out, but still has a wide mediolateral extension. At the meso-diencephalic junction these disseminated



Fig. 4 Semidiagrammatic representation of the periventricular CA fibre system (rostral to the locus coeruleus), the medial fibre flow of the tegmental CA radiations (TR), and the CA fibres of the mammillary peduncle (MP). Composite drawing of somewhat different paramedian sagittal planes. For abbreviations, see Index.

CTT fibres bend ventrally through, and partly lateral to the medial lemniscus to run broadly into the zona incerta and Forel's H₅-field (Fig. 2). Here they run in a position and follow a course very similar to the myelinated fascicles of the incerto-tegmental and incerto-tectal tracts, as defined by e.g., Gurdjian²⁰ Bürgi and Bucher¹² and König and Klippel²¹. Ventrally there is no clear delineation between these CTT fibres in the zona incerta and the outflow of fibres from the ventrolateral part of the tegmental radiations and the mesencephalic A 8 cell group as described in more detail below. In the rostral mesencephalon and the caudal diencephalon, the lateral parts of the CTT pass along the crus cerebri and fibres leave the system ventrolaterally. The most caudal ones run around the dorsal aspect of the crus cerebri, and the more rostral fibres run through the crus cerebri, towards the deep inner surface of the optic tract, into the supraoptic decussations. Further rostrally at the level of the subthalamic nucleus, remaining CTT fibres bend ventrally and laterally to run — partly intermingled with the internal capsule branch of the DTB (see above) — through the internal capsule towards the ansa lenticularis and the supraoptic decussations. These fibres might also reach the neostriatum and possibly the overlying cortex (cf. Fig. 16).

C. THE DORSAL PERIVENTRICULAR SYSTEM

The GA method revealed a prominent CA fibre system extending in the periventricular and periaqueductal gray of the medulla oblongata, pons, mesencephalon and diencephalon. It can be regarded as an adrenergic component of the dorsal longitudinal fasciculus, as described by Crosby and Woodburne²² and Nauta and Haymaker²³. The most caudal fibres of the system were observed in a superficial position at the level of the nucleus of the solitary tract and the dorsal vagal nucleus. The fibres could be followed from this area, which is heavily supplied with fluorescent CA terminals and contains CA cell bodies belonging to the A 2 cell group as a rather sparse bundle running rostrally underneath the ependyma of the fourth ventricle. The direction of these fibres has not been established, it seems that they either could be axons ascending from the A 2 cell group or could represent descending fibres from the locus coeruleus cell group. At the level of the locus coeruleus, the system increases considerably in width and in the number of fibres, in that the locus coeruleus and perhaps also other systems (e.g., the CA cell bodies distributed along the superior cerebellar peduncle group A 4) contribute ascending fibres to the system. The details of the arrangement of the DPS fibres in this region are obscured by the presence of a dense fluorescent terminal supply to the locus coeruleus and adjacent periventricular gray matter. As noted above, it seems highly probable that these terminals arise from dorsally directed collaterals of the ascending CTT fibres. From the region dorsal and medial to the locus coeruleus, the DPS ascends rostrally underneath the ependyma occupying the lateral part of the periventricular gray. Among the fibres are scattered CA-containing cell bodies that in

sagittal sections appear as a diffuse rostromedial extension of the locus coeruleus CA cell group. Supposedly these cells also project into the DPS.

Along its course underneath the fourth ventricle the DPS gives off fibres medially and in frontal sections, a continuous flow of fibres was observed crossing the midline just beneath the ependyma. It seems probable that these crossing fibres represent a broad pontine decussation of the DPS fibres. When these medial fibres reach the midline some of them turn sharply ventrally to run ventrorostrally along the raphe following a course parallel to the caudal surface of the decussation of the superior cerebellar peduncles ventrally they assume a rostral direction to ascend through the ventromedial tegmentum close to the interpeduncular nucleus. The course of this median pathway appears to be similar to that of the medial tegmentomammillary and the tegmentopeduncular tracts described by Ban and Zyo³ in the rabbit and Briggs and Kaelber¹⁰ in the cat. Somewhat more laterally (Fig. 4) these fibres were paralleled by another branch from the DPS running from the region of the locus coeruleus ventrorostrally through the lateral part of the medial longitudinal fasciculus. These fibres apparently give rise to a dense terminal network in the ventral tegmental nucleus, and then continue their ventrorostral course behind the decussation of the superior cerebellar peduncles. Like the median fibres they gradually bend rostrally to ascend — partly through the decussation — into the ventromedial tegmentum (Fig. 4). Interestingly this more lateral branch of the DPS follows a course that is conspicuously similar to that of the mammillary peduncle as described with classical neuroanatomical techniques^{10, 12, 17, 51, 52}. In addition to ascending fibres, it seems possible that the described CA pathways could comprise also descending fibres, originating in cells of the ventromedial tegmentum.

At the rostral end of the fourth ventricle, some of the DPS fibres sweep dorsally around the ventricular edge to run in a dorsocaudal direction into the posterior colliculus (Figs. 4 and 6). The laterally situated fibres in the DPS run rostrally along the mesencephalic root of the trigeminal nerve up to the decussation of the superior cerebellar peduncles. They run above and through the dorsal part of the superior cerebellar peduncle and turn ventrorostrally on its rostral side to join the so-called tegmental radiations (see below). The medial part of the DPS runs rostrally into a tangle of fluorescent fibres situated in the rostral part of the dorsal raphe nucleus and in the adjoining ventrolateral central gray. This tangle appeared to comprise not only irregularly running nonterminal axons and scattered CA-containing cell bodies but also a terminal axonal network that seemed to arise from collateral branches of the ascending CTT fibres (see above). In the intact animal, it was not possible to trace the DPS fibres through this region, but there occur three major outflows from the fibre tangle that might at least partly represent the further continuation of the caudal part of the DPS. One outflow is dorsocaudally towards the posterior colliculus, located immediately rostral to the previously mentioned collicular projection from the DPS. A second outflow is ventrorostrally along the tegmental radiations. The third gives rise to a well-defined bundle running rostrally in the ventrolateral part

of the periaqueductal gray within the dorsal longitudinal fasciculus. This bundle named the dorsal periventricular bundle (DPB) is described in more detail in a parallel communication.²⁷ As illustrated in Fig. 4 the DPB — after having given off fibres to the anterior colliculus — gives rise to a diencephalic periventricular system that projects to medial and midline thalamic epithalamic pretectal and hypothalamic regions. As described below the hypothalamic branch of the DPB most probably contributes to the so-called ventral periventricular system. The lesion experiments of Lindvall *et al.*²⁷ provide evidence that the DPB axons have their origin primarily in cell bodies (defined as the A 11 group) located among the DPB fibres and distributed along the periaqueductal and periventricular gray of the mesencephalon and the caudal thalamus (Fig. 5).

D THE TEGMENTAL CATECHOLAMINE RADIATIONS

The term tegmental radiations (*radiatio grisea tementi*) was used by Wetsche del²⁸ and Kuypers²⁹ to describe the almost radially-oriented systems of unmyelinated or scarcely myelinated fibres in the mesencephalic tegmentum. These fibres are oriented in the frontal plane and when going from dorsal to ventral they diverge strongly in the ventrolateral direction into a fanlike arrangement. When going from caudal to rostral the frontal planes formed by the radiating fibres converge strongly ventrally into the region of the rostral, ventral mesencephalon (see ref. 3).

The tegmental radiations represent an architectonical principle in the mesencephalon that is of central importance for the understanding of the arrangement of the ascending CA fibre systems. Major portions of the medullary pontine and mesencephalic fibre systems follow the tegmental radiations to assemble at the meso-diencephalic junction to form the ascending medial forebrain bundle and ventral periventricular CA systems. As pointed out above all previously described ascending CA fibre systems — the DTB, the CTT and the DPS — and also mesencephalic CA cell groups, project through the tegmental radiations. Although CA fibres run ventrally and ventrolaterally along the tegmental radiations at all rostrocaudal levels of the mesencephalon (Fig. 1), the massive flow of fibres occurs in a rather narrow section, lying in between the decussation of the superior cerebellar peduncles and the red nucleus. This dense mesencephalic CA fibre arrangement will be referred to as the tegmental CA radiations, and it is illustrated in Fig. 7 and 8 in an oblique frontal section falling in the plane of the radiating fibres, immediately rostral to the decussation of the superior

Fig. 5 Photomontages of the dorsal and ventral periventricular CA fibre systems in a horizontal and a paramedian sagittal section, respectively. *Above:* the dorsal periventricular bundle in the periaqueductal gray of the mesencephalon (AQ=aqueduct). Note the fluorescent cell bodies with a somewhat diffuse outline that project fibres rostrally (to the left) along the bundle. (X 190). *Below:* The ventral periventricular system ascending broadly along the lateral aspect of the periventricular hypothalamic nucleus, between the dorsomedial (dmh) and the paraventricular (pvh) nuclei. These latter nuclei are heavily supplied with fluorescent CA terminal networks. Cf. Fig. 4 (X 100).

AO

dmh

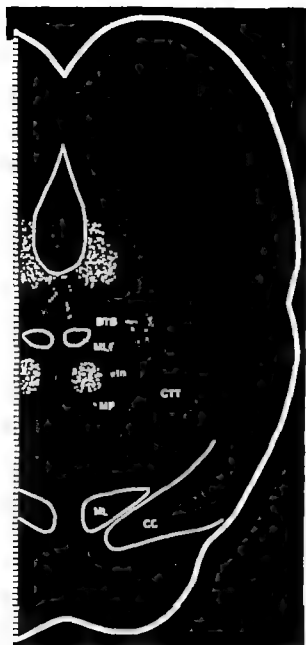


Fig. 6. Semidiagrammatic representation of the position of the ascending CA fibre systems in the dorsal tegmental bundle (DTB), the central tegmental tract (CTT), and the mamillary peduncle (MP) in a transverse section at the level of the ventral tegmental nucleus (vtn). For further abbreviations, see Index.

cerebellar peduncles. From the position of the radiating fibres, a median, a medial, and a lateral flow can be distinguished.

The median CA fibre flow is within and along the raphe. The fibres are very

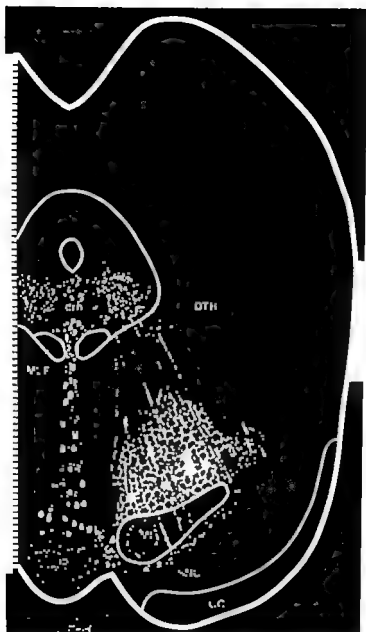


Fig. 7 Semidiagrammatic representation of the tegmental CA radiations in an oblique frontal plane, falling just rostral to the decussation of the superior cerebellar peduncles. Cf. Figs. 1, 2, and 4. For abbreviations, see Index.

delicate with fine spherical varicosities. They can be traced from the fibre tangle in the rostral part of the dorsal raphe nucleus in a rostroventral direction, between the two medial longitudinal fasciculi, down to the region dorsal to the interpeduncular nucleus, where most fibres are seen to turn sharply rostrally



Some of the fibres enter this nucleus from the dorsal side. Possibly some of the median fibres originate in the DPS but it seems highly probable that the most substantial contribution comes from the CA-containing cell bodies distributed along the raphe and located within the median fibre flow (the dorsal cells of the A 10 group). It is notable that an aggregation of these cells was located in the ventral portion of the dorsal raphe nucleus, a nucleus that is otherwise known to contain many indolamine-containing cell bodies.^{9, 10}

The medial flow of fibres runs in a ventrolateral (and somewhat rostral) direction from the fibre tangle in the dorsal raphe region in a position just lateral to the medial longitudinal fasciculus and medial to the DTB. Many of these fibres are assembled into strongly fluorescent fibre aggregates that split up into a characteristic network as they reach the ventral part of the tegmentum. Most probably part of these fibres terminate as strongly fluorescent varicose fibres in this region.

The lateral fibre flow is formed by the ventrolaterally turning DTB and CTT fibres, as described above. In sagittal sections, it was noted that part of the lateral fibres belong to a pathway running in the lateral part of the DPS from the locus coeruleus along the mesencephalic root of the trigeminal nerve turning ventrally in front of the superior cerebellar peduncle (cf. above). As illustrated schematically in Fig. 7 the lateral fibres run first ventrolaterally (and somewhat rostrally) into the region dorsal to the lateral part of the crus cerebri, where they might contribute to the adrenergic terminals present in this area. The laterocaudal group of mesencephalic CA cell bodies (the so-called A 8 group) is located in this ventrolateral part of the tegmental radiations, and the fibres of the lateral flow thus intermingle here with the outgoing fibres of the A 8 group. The further course of these axon systems was analysed in serial pseudo-sagittal sections, oriented about 45° to the sagittal plane. As already pointed out by Kuypers²¹ this plane of section yields very valuable information on the further rostral course of the radiating fibres. It was thus revealed that the CA fibres of the lateral flow turn medially in the ventrolateral part of the tegmental radiations and intermingle with the axons of the A 8 cells. They run in medial and rostromedial directions to reach the ventral tegmental area of Tsai and the MFB on the dorsal and ventral side of the medial lemniscus. The more caudally situated fibres run straight medially dorsal to the medial lemniscus, and the more rostral fibres run rostromedially in between the medial lemniscus and the substantia nigra through the caudal zona incerta and the Hs-field of Forel, to join the MFB system in the caudal hypothalamus.

From the above description it is evident that there is a massive convergence

Fig. 8 Details of the CA fibre flows in the tegmental CA radiations photographed in the oblique frontal plane illustrated in Fig. 7. A. The median fibre flow in its position between the two medial longitudinal fasciculi. (X 260). B and C. The medial fibre flow ventromedial to the DTB (X 225 and 240, respectively). D. Detail of the fibre arrangement in the ventrolateral part of the tegmental radiations (medial is to the left of the picture). (X 140).

of the CA fibres from the tegmental radiations at the meso-diencephalic junction in the region medial to the substantia nigra. Here together with fibres from the ventrally situated A 10 cells and probably also from some of the CA cells in the substantia nigra (group A 9), the converging fibres give rise to the ascending CA fibre systems within the MFB and the VPS (described below). Fibres also project to the mamillary complex, which in the GA material was revealed to have a much richer and more complex innervation than was previously observed with the Falck Hillarp technique. Three pathways were seen to contribute in this innervation. One is a direct rostral projection of very thin varicose fibres running superficially just above the interpeduncular fossa. Dorsal to the posterior mamillary nucleus, these fibres turn sharply ventral to give rise to a rich plexus of delicate varicose terminals in this nucleus. The second mamillary pathway appeared to arise from fibres situated more dorsally in the rostral projection from the median fibre flow. These delicate fine varicose fibres run rostrally in between the fibres of the ventral tegmental decussation (dorsal to the interpeduncular nucleus) past the fasciculus retroflexus on the medial side and down into the medial mamillary nucleus along the nonfluorescent myelinated fibres of the mamillotegmental tract. A third pathway to the mamillary complex is probably along the mamillary peduncle. As described above a branch from the DPS runs, from the region of the dorsal and ventral tegmental nuclei rostromedially along the course of the mamillary peduncle. These fibres could not be traced through the dense fibre arrangements in the ventromedial tegmentum but at a more rostral level in its well-defined position in the mamillary region the peduncle was again observed to comprise many CA axons. They probably reach the mamillary nuclei from the lateral side and some of them may continue rostrally in the MFB system. The two latter pathways appear to be the main sources for the CA innervations of the medial and lateral mamillary nuclei. In addition the VPS was observed to give fibres to the medial nucleus as described below.

E. THE VENTRAL PERIVENTRICULAR SYSTEM

Caudally this CA fibre system was distinguished in the region immediately dorsolateral to the interpeduncular nucleus. The fibres run rostrally ventral to the supramamillary commissure and then medial to the mamillothalamic tract. In passing above the medial mamillary nucleus, fibres are seen leaving the bundle into the nucleus. Behind, through, and rostral to the mamillothalamic tract fibres from the medial part of the MFB leave this bundle partly as collaterals, rostromedially to join the VPS. The VPS thus increases in width and fibre number as it passes in a rostral and somewhat dorsal direction through the posterior hypothalamic area into the dense CA terminal area of the dorsomedial hypothalamic nucleus. Here the fibres meet those of the hypothalamic branch of the DPB which in all probability at least partly join the VPS, and form a broad ascending hypothalamic CA fibre system dispersed on the lateral aspect of the periventricular nucleus (Fig. 4). In paramedian sections,

the VPS could thus be seen to form a connexion between the CA terminal systems of the dorsomedial and the paraventricular hypothalamic nuclei (Fig. 5). It seems possible that also the MFB system could contribute to this part of the VPS via the branches of the MFB that project into the dorsomedial and the paraventricular nuclei (Fig. 10 see below). Rostral to the paraventricular nucleus (Figs. 4 and 10), the VPS becomes dispersed mediolaterally, and some of the medial fibres turn dorsally to continue as periventricular thalamic fibres. Most of the VPS fibres form a broad band that sweeps dorsally on the caudal and rostral sides of the anterior commissure, into the caudal septum and possibly also into the fornix towards the hippocampus. The most lateral part of the VPS runs rostrolaterally into the medial part of the CA terminal system in the Interstitial nucleus of the stria terminalis. Here these fibres intermingles with fibres coming from the MFB (see below). Also these lateral VPS fibres might continue into the septum, running dorsally on the caudal and rostral surfaces of the anterior commissure. It is conceivable that other fibres could turn ventrolaterally to reach the MFB system. It has not yet been established, however whether the VPS projects further rostrally. Nor has its terminal distributions within hypothalamus and septum been mapped. However it seems reasonable to suppose that the VPS contributes primarily to the CA innervations in the medial and periventricular hypothalamic areas and in the caudal septum. The existence of the periventricular CA fibre system, which has not been previously demonstrated, introduces new aspects on the organization of the hypothalamic and septal CA afferent systems. This will be pursued in more detail in further studies.

F THE MEDIAL FOREBRAIN BUNDLE SYSTEM AND THE NIGROSTRIATAL PATHWAY

Within the MFB a highly heterogeneous system of CA fibres ascends from the brain stem reticular formation towards diencephalic and telencephalic regions. As described in the previous paragraphs, both the DTB and the CTT have major routes of projection along the MFB and it is possible that also the DPS contributes fibres to this system. Another source of ascending CA fibres in the MFB is the mesencephalic CA cell groups, primarily the A10 group, forming the so-called mesolimbic DA system¹⁻⁶⁴. Closely associated with the MFB system is also the nigrostriatal DA pathway which from previous studies^{1, 14, 43, 47-50, 64} is known to originate in the pars compacta of the substantia nigra (group A9), and possibly also in cells located in the ventral tegmental area of Tael (A10) and in the more caudally situated A8-group. Moreover there is recent evidence⁶ that the substantia nigra DA cells project also along the MFB to innervate the anterior limbic cortex. The axons of the nigrostriatal and mesolimbic pathways were identified with an extremely delicate fluorescent axon type equipped with fairly regularly spaced, fine varicosities, which were characteristically arranged in fascicles. This characteristic fluorescence morphology of these presumed DA-containing axon systems made it possible to distinguish them from other CA axon systems running in the MFB. They could thus be traced from the

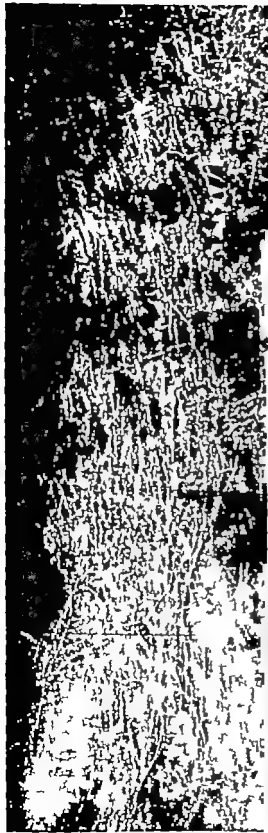
mesencephalic CA cell groups all the way up to their terminal areas, here the morphology of the axons changed as they ramified into terminal and paraterminal branches.

Caudally the CA fibre systems of the MFB and the nigrostriatal pathway assemble at the meso-diencephalic junction in the area dorsal to the mamillary peduncle and medial to the substantia nigra. The fibres assemble from the tegmental CA radiations, as described above via its strongly converging rostral outflow which carries fibres primarily from the medullary and pontine components of the CTT from the mesencephalic CA cell groups, and to a minor extent probably also from the DTB and the DP. It seems also possible that fibres running along the mamillary peduncle (see above) could continue rostrally in the MFB system in agreement with findings obtained with stains of anterograde degeneration (e.g. 81). Further rostrally the DTB joins the MFB in the middle hypothalamus, and at this level the ascending CA fibre system reaches its maximum thickness and abundance. The fluorescence morphology of the various CA axon systems allowed a rather safe distinction between the different ascending systems (cf. ref. 35), and their relative position in the bundle could therefore be observed. Interestingly the DTB and the CTT fibres retain in the MFB their relative positions in the pontine and mesencephalic tegmentum: the DTB fibres running in the dorsomedial part of the MFB and the CTT fibres running ventrally and laterally to them. The mesencephalic (probably DA-containing) MFB fibres occupy a lateral and somewhat dorsal position and they adjoin dorsally the more compact and fibre-rich nigrostriatal bundle which runs in a position immediately dorsolateral to the MFB along the medial edge of the crus cerebri and the internal capsule.

1 Presumed dopamine-containing systems

The nigrostriatal pathway (NSP) (Figs. 9 and 10). The fibres from the CA cells in the pars compacta of the substantia nigra are in their initial course directed medially. Medial in the substantia nigra, they turn sharply rostrally and assemble in a well-defined bundle that ascends in the H-field of Forel immediately dorsolateral to the MFB system. The most dorsal fibres in the NSP leave the bundle first. At the level of the subthalamic nucleus, these dorsal fibres bend first sharply laterally above the crus cerebri, and then rostrally to turn into the internal capsule from the caudal side towards the caudal parts of the neostriatum. The somewhat more ventrally located fibres deviate less sharply and run in a

Fig. 9 Left Photomontage of the medial forebrain bundle in a horizontal section similar to that illustrated in Fig. 10. The picture covers the bundle from the mamillary region (bottom) up to the level of the paraventricular hypothalamic nucleus (top). The branch projecting into the dorsomedial nucleus is seen to the right (λ 85). *Right* Sagittal section through the rostral hypothalamus showing DTB fibres in the dorsomedial part of the medial forebrain bundle. The fibres are seen to give off abundant collateral branches into the supraspinal decussations and towards the reticular thalamic nucleus and the stria medullaris (cf. Fig. 1). (X 160).



rostromedial direction through the subthalamic region into the internal capsule. The centrally and ventrally located fibres continue rostrally along and partly within the dorsomedial edge of the internal capsule. Along this course the more dorsally situated fibres in the bundle deviate in a rostro-latero-dorsal direction into the internal capsule towards the central parts of the nucleus caudatus-putamen. The ventral portion of the NSP continues further rostrally up to the level of the globus pallidus. Here most of them fan out in rostromedial and dorsal directions to run along the myelinated fascicles through the globus pallidus into the head of the nucleus caudatus-putamen. The most ventral portion of the NSP however continues rostrally in a position just dorsal to the MFB up to the anterior commissure; they pass ventrally to the commissure and medially and laterally to its anterior limb; they run into the nucleus caudatus-putamen and the dorsal part of the interstitial nucleus of the stria terminalis. The ventral portion of the NSP also contributes fibres to the ansa lenticularis, as described below.

The MFB systems. The presumed DA-containing fibre systems in the MFB run in a position immediately ventromedial to the NSP and they are not well demarcated from each other. The several ascending DA systems appear rather as a single large bundle whose ventromedial portion runs within the MFB as this tract usually is defined in the classical neuroanatomical literature (see e.g., ref. 33). The MFB fibres run in this position up to the level of the retrochiasmatic region where fibres leave the bundle laterally to run together with the fibres coming from the most ventral position of the NSP system *within the ansa lenticularis* — intermingled with presumed NA fibres — towards the amygdala, the caudal part of the ventral neostriatum, and the piriform cortex. It is notable that many of these DA fibres were observed to be collaterals from axons continuing rostrally. The MFB system then separates into different components: One deviates slowly dorsally to run — dorsal to the MFB — in a rostral and somewhat dorsal direction *into the nucleus accumbens*. A second component takes a ventral position in the MFB. In horizontal sections it was observed that these ventral fibres fan out in rostromedial and rostromedial directions in the region just dorsal to the olfactory tubercle and from here turn ventrally *into the olfactory tubercle*. Remaining fibres — constituting a major component of the system of presumed DA fibres in the MFB — continue rostrally within the MFB. Along its course this bundle gives off fibres that, together with presumed NA fibres, run in a dorsal and somewhat rostral direction *along the diagonal band into the septum* giving rise to a dense terminal system in the medial part of the lateral septal nucleus.

A minor portion of the bundle continues rostrally within the MFB up to the anterior part of the nucleus accumbens. Here the fibres run dorsally along the rostromedial aspect of the accumbens and pass medially and laterally to the anterior limb of the anterior commissure. These fibres follow the external capsule, where they run in dorsal and lateral directions out on the surface of the nucleus caudatus-putamen and into the deep layers of the frontal cortex. CA fibres were seen to run in the external capsule also at more caudal levels; they seem to project both into the neostriatum and into the piriform cortex.

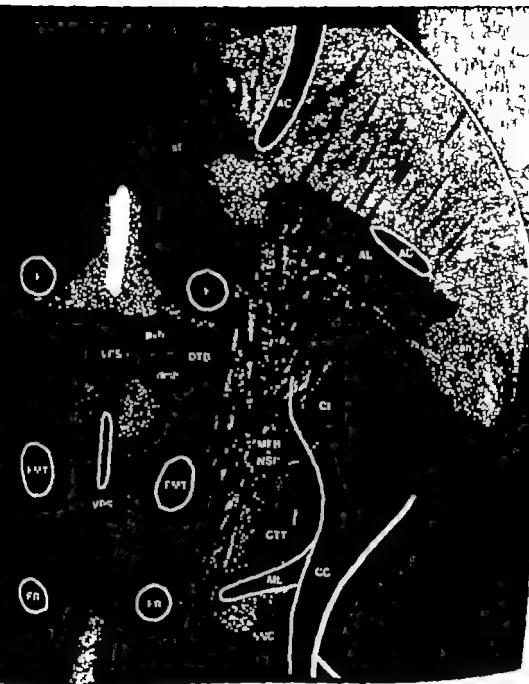


Fig. 10 Semidiagrammatic representation of a horizontal section through the dorsal part of the medial forebrain bundle system (MFB), the ventral part of the nigrostriatal pathway (NSP), and the ansa lenticularis (AL). Compare with the sagittal drawings in Figs. 1 and 2 and the frontal drawing in Fig. 12. Composite drawing of slightly different horizontal planes. For abbreviations, see Index.

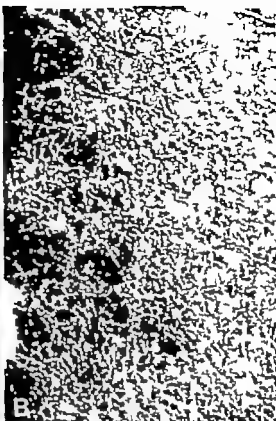
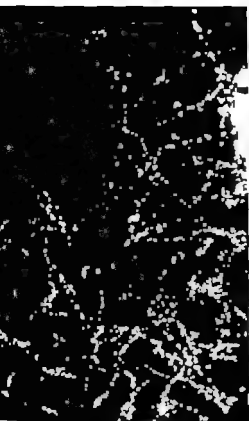
The major portion of the bundle leaves the MFB at the level of the rostral septum in a dorso-medio-rostral direction to run in a position well corresponding to the septohypothalamic tract. The fibres sweep as a broad band along the medial and the medioventral aspects of the nucleus accumbens. The dense DA terminal system in this nucleus was not sharply demarcated from the bundle probably signifying a ramification from the bundle into the nucleus. In the region rostromedial to the nucleus accumbens the bundle separates into four main branches. a) The branch of greatest abundance runs dorso-rostrally and laterally *into the deep layers of the frontal cortex* mingling with the above mentioned fibres passing rostrolaterally to the accumbens. These fibres give rise to an extensive DA-containing terminal system in the frontal cortex & \times (Fig. 11). The branch to the frontal cortex passes along the rostral aspect of the external capsule and fibres are seen to run *into the external capsule* thus contributing to the above mentioned CA fibre system within the external capsule. b) A second branch of the bundle turns more sharply dorsally to run in a position caudal to the branch to the frontal cortex. It sweeps caudally above the corpus callosum *into the anterior limbic cortex* medial to the cingulum. As described in more detail by Lindvall *et al* \times and Björklund *et al* \times these fibres give rise to an abundant dopaminergic terminal system in the anterior limbic cortex. c) A third branch runs in a dorso-caudal direction *into the septum* contributing to the innervation of this area. This branch comprised both collaterals and fibres deviating from the bundle. d) Fibres in a ventral position in the bundle turn first dorsally for a short distance and then rostrally to run *within the medial olfactory tract*. Presumably these fibres give rise to terminals in the olfactory nuclei.

Presumed noradrenaline-containing systems

Probably reflecting their highly diversified origins, the morphology of the presumed NA axons in the MFB is quite heterogeneous. The locus type of preterminal axon reaching the MFB via the DTB and possibly also via the CTT (see above) has a characteristic appearance and can thus be identified in the fluorescence microscope. The non-locus types, on the other hand, consist of headed fibres of much varying appearance and widely varying calibres. In the following description the different morphological types of non-locus fibres therefore will not be distinguished (cf Fig. 9).

The preterminal axons of the *locus type* run predominantly in the dorsomedial part of the MFB. In the rostral hypothalamus, the axons give off abundant

Fig. 11 Details of the two principal non-locus types innervating the neocortical areas. A. Varicose terminals of the locus coeruleus, presumed noradrenaline-containing type in the molecular layer of the sensory-motor cortex. ($\times 400$). B. Presumed dopamine-containing axon terminals in the deep layers of the frontal cortex. ($\times 460$). C. Nonterminal axons of the locus coeruleus type in the rostral part of the septum running towards the cingulum. ($\times 350$). D. Nonterminal presumed dopamine-containing axons running on the rostral side of the neostriatum (whose dense fluorescent terminal network is seen to the right in the picture) towards the frontal cortex. ($\times 350$).



collaterals ventrally and dorsally. Of the dorsally directed collaterals the caudal ones run into the reticular thalamic nucleus, and the more rostral collaterals form a bundle which runs on the dorsal surface of and partly also within the *stria medullaris*. This bundle contributes to the adrenergic innervation of the anterior thalamic nuclei. The ventrally directed collaterals mix with collaterals of the non locus axons to run into the *suprapile decussations* (see below).

Somewhat further rostrally locus and non-locus fibres leave the MFB dorsally into the ventral part of the *interstitial nucleus of the stria terminalis* which is heavily supplied with fluorescent CA-containing varicose fibres. They represent to a large extent at least the ramifications of the non locus fibres (refs. 55 & unpublished observations) whereas the locus fibres appear to pass through the nucleus into the *stria terminalis the fornix* and the *caudal septum*. Locus fibres were also seen to run medially across the midline along the ventral surface of the anterior commissure. Within the *stria terminalis*, the axons were followed all the way to the amygdala. Dorsally when passing close to the head of the nucleus caudatus-putamen at the level of the fornix commissure fluorescent fibres were seen to leave the *stria terminalis* rostrally through the internal capsule into the caudate nucleus. The locus fibres running in the fornix were traced into the hippocampus.

Remaining locus fibres continue rostrally in the MFB up to the level of the rostral septum. Here some fibres continue rostrally in a position ventrolateral to the intermediate olfactory tract into the lateral part of the anterior olfactory nucleus. The major portion however turns dorso-medio-rostrally to run close to the previously described fibres of the DA-containing type along the septohypothalamic tract (Fig. 11C). Just ventral to the genu of the corpus callosum, the bundle of locus fibres divides into a caudal and a rostral branch. The caudal branch was traced underneath the corpus callosum in the *fornix superior* all the way back to the hippocampus. The rostral branch runs caudally above the corpus callosum within the *cingulum*. This branch gives off fibres along its course to large areas of the neocortex, and caudally it sweeps around the splenium of the corpus callosum to enter the hippocampus from the caudal side.

The preterminal fibres of the *non-locus* types occupy a position in the MFB ventral and lateral to the locus fibres. Along their course through the hypothalamus and the preoptic region they give off abundant collaterals that contribute importantly to the adrenergic innervations of these regions.

a) From the most ventral part of the MFB a loosely arranged system of collaterals leaves the bundle in a ventromedial direction along the ventral brain surface towards the *medlobasal hypothalamic area*. Part of these fibres most probably enter the median eminence constituting the so-called *reticulo-infundibular NA pathway* as described by Björklund *et al* *

b) Abundant collaterals of non-locus fibres leave the MFB system medially at two levels in the hypothalamus (Fig. 10). First, broadly into the *dorsomedial nucleus* and second as a well-defined branch that projects medially and somewhat rostrally from the dorsal part of the MFB into the *paraventricular hypo-*

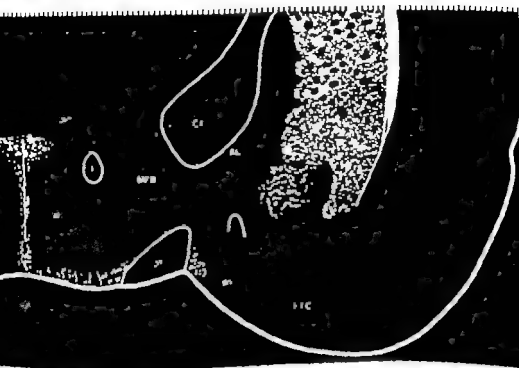


Fig. 12. Semidiagrammatic representation of the CA fibre systems running in the supra-optic decussation (SOD) and the area lenticularis (AL) as observed in a frontal section through the retrochiasmatic region. Cf. Fig. 10. Composite drawing of slightly different frontal planes. For abbreviations, see Index.

thalamic nuclei. They most probably contribute importantly to the dense adrenergic innervations of these nuclei.

c) At the level of the retrochiasmatic region, abundant fibres leave the MFB ventromedially to pass on both sides of the fornix into the supraoptic decussation (SOD). The highly interesting arrangement of these projections is illustrated semidiagrammatically in Figs. 12 and 13. The fibres decussate — intermingled with the decussating locus fibres — within the SOD and along their course they give off collaterals dorsomedially into the anterior hypothalamic area, dorsally into the anterior periventricular nucleus (Fig. 12), rostrally as a broad band of fibres past the optic chiasm on its dorsal aspect (Fig. 13), and probably to a minor extent also caudally into the retrochiasmatic region. In animals subjected to a total unilateral destruction of the ascending pontine and medullary CA fibre systems (unpublished observations) it was revealed that these collaterals were given off from the decussating fibres on both sides of the midline. The most medial of the rostrally directed fibres run medial to the suprachiasmatic nucleus into the periventricular preoptic region. The more lateral fibres fan out in the rostral and rostralateral directions, giving rise to axonal ramifications in the ventral part of the preoptic area and in the supraoptic nuclei. Some fibres sweep just rostral to the supraoptic nucleus out laterally into the piriform cortex. The

decussating fibres continue laterally and caudally within the SOD along the dorsomedial surface of the optic tract. Part of the fibres deviate laterally into the ansa lenticularis (see below) and others turn laterally and ventrally into the posterior part of the supraoptic nucleus (Fig. 12). Fibres, primarily of the locus type were traced along the optic tract all the way back to the region of the metathalamus. Although their direction was not established it seems probable that part of them represent caudally running axons from the SOD contributing to the innervation of the geniculate bodies. In addition to the inflow from the MFB CTT and DTB fibres reach the SOD also from the zona incerta through the internal capsule and caudally from the lateral midbrain tegmentum as pointed out above but their courses and terminations were not established.

d) A major projection of the MFB system is along the *ansa lenticularis*. As illustrated in Figs. 10 and 12, part of these fibres are aggregated into a rather dense bundle which leaves the MFB in a lateral direction at the level of the retrochiasmatic region. The bundle comprises fibres of both the DA and the non locus NA types many of them being identified as collaterals of the fibres continuing rostrally within the MFB. The bundle ran laterally within the ansa lenticularis — receiving further fibres coming from the SOD and also from the zona incerta through the internal capsule (cf. above) — into the caudal part of the ventral neostriatum and the amygdala (particularly the central amygdaloid nucleus).

Further rostrally in the suprachiasmatic and preoptic regions, there is a lateral flow of loosely arranged disseminated fibres, primarily coming from the ventral part of the MFB and from the rostrally projecting collaterals of the SOD (cf. above). The course and position of this disseminated fibre path correspond well to the so-called amygdalofugal pathway as described e.g. by Valverde²² and Nauta and Haymaker²¹. The CA fibres in this pathway run laterally and caudally to project broadly into the amygdaloid piriform region. The most medially located fibres could be seen to enter the subiculum of the hippocampus, signifying another route of projection of the CA afferents to the hippocampus.

e) Somewhat further rostrally non locus fibres leave the MFB dorsally and slightly medially into the ventral part of the *interstitial nucleus of the stria terminalis* together with fibres of the locus type as mentioned above. The non locus fibres ramify abundantly within the nucleus, but it seems possible that they could continue past the anterior commissure into the septum. In between the two interstitial nuclei fibres cross the midline along the ventral and caudal surfaces of the anterior commissure partly intermingled with the above-mentioned decussating locus fibres. Decussating fibres, possibly belonging to this system were also detected within the anterior commissure.

f) In the preoptic region fibres leave the MFB in medial and ventromedial directions into the *medial preoptic area* and along the *diagonal band* towards the *septal nuclei* (Fig. 13).

An extension of non-locus fibres beyond the preoptic and septal regions uncertain from the present observations. It seems possible however that

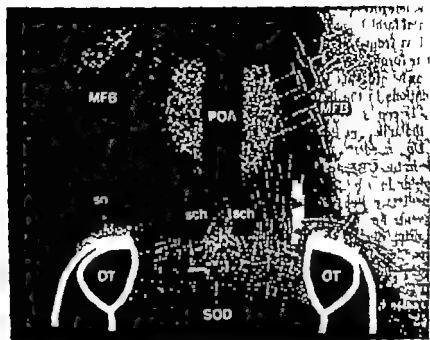


Fig. 13 Semidiagrammatic representation of the CA fibres in the supraoptic decussations (SOD) and their rostrally and caudally projecting collateral branches as seen in a horizontal section falling immediately dorsal to the optic chiasm. Fibres with abundant collaterals running in the most ventral part of the MFB system are also seen in the preoptic region. For abbreviations, see Index.

fibres could reach, e.g. the olfactory bulb but this can only be verified through further experimental work.

DISCUSSION

The ascending CA fibre systems from the mesencephalic, pontine, and medullary reticular formation are principally associated with four major and important conduction pathways, the *periventricular fibre system*, the *central tegmental tract*, the *medial forebrain bundle system*, and the *nigrostriatal pathway*. To a very large extent the adrenergic pathways follow — along or reciprocal to — well established non-adrenergic fibre tracts. Thus a number of fibre systems, well known from the classical neuroanatomy carry also adrenergic axons, for instance the *fasciculus retroflexus*, the *mammillo-tegmental* and *mammillo-thalamic tracts*, the *stria medullaris*, the *stria terminalis*, the *fornix*, the *mammillary peduncle*, the *ansa lenticularis*, and the *supraoptic decussations*. Therefore it seems both feasible and desirable to adapt as far as possible the nomenclature and description of the CA systems to the prevailing neuroanatomical terminology.

The central tegmental tract (CTT) offers some special problems in this respect. In the neuroanatomical literature, the CTT is usually identified with a highly heterogeneous and partly loosely arranged system of longitudinally running fibres in

the reticular formation. The tract, whose fibres follow the characteristic myelinated tegmental fascicles, comprises both ascending systems from the reticular formation as well as from the spinal cord ^{45, 52, 53} and several descending systems to the reticular formation ^{12, 13, 44, 64}. The ascending adrenergic fibres in the CTT have a similar heterogeneous and partly loose arrangement. On the basis of origins and terminations in the brain, the CA fibre system, designated CTT in the present paper is composed of many different axonal pathways (e. g. ponto-cortical, ponto-thalamic, ponto-hypothalamic, ponto-tectal, bulbo-hypothalamic fibres, etc.). Ungerstedt ⁶¹ and Olson and Fuxe ⁶⁵ distinguished a dorsal and a ventral bundle in the CTT and Maeda and Shimizu ⁴² and Maeda et al. ⁴¹ made a further subdivision into a dorsal pathway (originating in the principal locus coeruleus), an intermediate pathway (originating in the disseminated pontine CA cell bodies), and a ventral pathway (originating in the medullary cell groups). This subdivision, made primarily on the basis of the connexions of the fibres, did not have a correspondence in distinguishable separate bundles in the present material. In agreement with Ungerstedt's ⁶¹ description, the fibres in the dorsomedial part of the CTT system were characteristically aggregated into a so-called dorsal bundle (the DTB) but this bundle did not carry all CTT fibres originating in the principal locus coeruleus. Remaining parts of the CTT have a more complex organization involving a considerable overlap between the pathways originating in the disseminated pontine CA cells, and in the medullary cell groups. These pathways should therefore be regarded as mutually overlapping components of the CTT system, as illustrated schematically in Fig. 16.

Also the MFB is highly heterogeneous with respect to CA fibres. This conduction pathway is a route of projection of fibres from practically all ascending NA and DA fibre systems. However as revealed in the present study the MFB is by no means the sole route of projection for the CA fibre systems to the di- and telencephalon. Thus, both the periventricular system, the CTT and the DTB — which partly contribute fibres to the MFB system — have important projection pathways also outside this system. It is also clear that the composition of the MFB, with respect to its CA fibres, differs considerably at different levels: in the posterior hypothalamus, in the anterior hypothalamus, or in the preoptic region.

THE PERIVENTRICULAR SYSTEM

This prominent CA fibre system — illustrated diagrammatically in Fig. 14 — is distributed along the periventricular and periaqueductal gray from the medulla oblongata up to the rostral diencephalon and the septal region. In the present description, a distinction is made between a dorsal system of fibres — extending along the dorsal longitudinal fasciculus, taken in its widest sense — and a ventral system extending along the periventricular region of the hypothalamus. The rostral, mesencephalic and diencephalic, part of the dorsal system forms a well-defined bundle the DPB that has previously been observed by Richardson and Jacobowitz ²⁹ in rats lesioned with 6-hydroxydopa. The DPB projects to medial

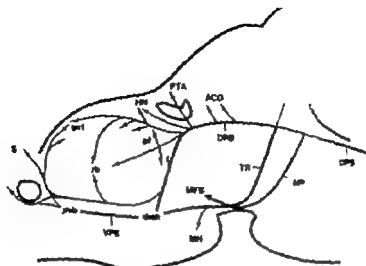


Fig. 14 Schematic representation of routes of projection of the periventricular and paramedian fiber systems to the tectum, diencephalon, and septum. Compare with Fig. 4 and text. For abbreviations, see Index.

and midline thalamic, epithalamic, pretectal, and hypothalamic regions along routes that fall essentially within the periventricular fiber system of Krieg.³⁰ For a more detailed account of the organization of the DPB system, the reader is referred to the paper by Lindvall *et al.*³⁷

In its arrangement the dorsal periventricular system (DPS) is quite different from the other CA systems studied, many of its cell bodies of origin being distributed diffusely along its extent through pons, mesencephalon, and the posterior thalamus. This signifies that axons are contributed to the bundle all along its extent in these regions. In addition the locus coeruleus was seen to project significantly rostrally along the DPS, and it seems possible that also the A4 and A2 cell groups and the CA cell bodies present in the area postrema³⁸ could contribute too. Moreover it seems possible that the DPS could contain also descending CA projections, particularly in the caudal parts of the system, but so far there are no observations to substantiate this assumption. The arrangement of the CA cell bodies in the DPS suggests that this adrenergic system — similar to what has been described for the non-adrenergic neuron systems in the dorsal longitudinal fasciculus^{12, 33} — may be composed of relatively short fiber systems that originate at different levels of the DPS and run for some distance along the bundle to terminate locally in the central gray or to leave the bundle at successively higher levels. With this arrangement it would seem improbable that any of the CA fibres would ascend in the DPS along its entire extent. In fact, Lindvall *et al.*³⁷ did not observe any significant removal of DPS fibres in the mesencephalic and thalamic parts of the system (i. e. the DPB) after a complete lesion of the DPS at the level of the dorsal raphe nucleus.

It is clear that the DPS carries prominent ascending CA systems projecting to tectal, pretectal, thalamic, epithalamic hypothalamic and possibly also septal regions. In addition the DPS together with the dorsal part of the mesencephalic A 10 cell group gives rise to a paramedian fibre system running rostroventrally along and lateral to the midbrain raphe probably contributing to the adrenergic innervation of the mamillary nuclei, to the ascending MFB system, and possibly also to the VPS. In part these paramedian mesencephalic CA fibres seem to constitute a component of the mamillary peduncle (cf. refs. 10, 13, 17, 51 and 52) which has been shown with classical neuroanatomical techniques to carry ascending fibres from the region of the dorsal and ventral tegmental nuclei to the mamillary nuclei 2, 10, 17, 45, 53. With stains for anterograde degeneration Nauta and Kuypers 53, Morest 45 and Briggs and Kaelber 10 have demonstrated a projection of the mamillary peduncle also further rostrally along the MFB to the lateral hypothalamic preoptic, and septal areas.

Ascending projections within the dorsal longitudinal fasciculus from the midbrain periaqueductal gray have been demonstrated with the Nauta and Fink Heimer methods by several authors 15, 26, 27, 45, 51, 52, 61. The routes of projection in the diencephalon of the ascending fibres, as revealed with these staining techniques, are notably similar to those of the adrenergic DPB described in the present paper (cf. ref. 37). It is thus evident that the adrenergic DPB system is a major component of the ascending dorsal longitudinal fasciculus. Constituting major connexions from Nauta's 50 "limbic midbrain area" the ascending periventricular fibre system and the mamillary peduncle have been presumed to play important roles in hypothalamic endocrine functions 50, 51, 61. The periventricular system has also been thought to be involved in such central functions as rage reactions, motor activity and food intake (for review of literature see Hamilton 59). Against this background the discovery of the prominent periventricular and paramedian CA fibre systems should be of great interest in the studies of the role of CAs in the functions of the reticular formation.

THE CENTRAL TEGMENTAL TRACT

As indicated above this term is used to describe the heterogeneous and partly loosely arranged longitudinal CA fibre system that courses with Forel's tegmental fascicles through the brain stem reticular formation. This fibre system comprises both ascending and descending pathways, and it has rich local projections within the lower brain stem and to the cerebellum that are not dealt with in the present description. On the basis of the fluorescence morphology of the CA axons, projections from the principal locus coeruleus and projections from the disseminated pontine and medullary CA cell groups could be distinguished.

Ascending projections from the locus coeruleus were observed along three routes: a) along the DTB, b) as disseminated fibres along the medial part of the CTT, and c) within the periventricular gray of the fourth ventricle, along the DPS. Some of the ascending locus fibres may cross the midline in the prominent decussation observed ventral to the nuclei. There was also a prominent descending

pathway running dorsomedially in the pontine and medullary portion of the CTT and from previous studies in other laboratories the locus coeruleus is known to project along the superior cerebellar peduncle to the cerebellum 18, 24, 25, 26. The DTB is the most abundant of the three ascending pathways, and its CA-containing axons seemed to be exclusively of the locus coeruleus type. In studies with the Falck Hillarp technique in conjunction with lesions, the DTB has previously been identified as an important projection route of the locus axons by Ungerstedt 24, Olson and Fuxe 24, 25, Maeda and Shimizu 42 and Maeda *et al.* 21. The latter authors observed in the cat also the projection from the rostral locus coeruleus along the DPS. Ascending reticular projections from the locus coeruleus region of the pons have also been observed with the Marchi stain for degenerating myelin by Russell 26 and Kuru and Yamamoto 21 in the cat. Interestingly these fibres, which can be presumed to be non-adrenergic in nature, run partly in a position that seems to be identical with that of the adrenergic DTB. Moreover from the studies of Bürgi and Bucher 12, 13 in the cat, it seems that also descending myelinated pathways (the so-called thalamo-preecto-tegmental fibres) run within this bundle. Thus, in its extension through pons and mesencephalon, the DTB appears to be a fairly well-defined, fibre-rich fascicle within the CTT carrying both ascending and descending, myelinated and non-myelinated fibre systems one of which is the ascending adrenergic locus projection.

In the GA-treated specimens, the dorsal tegmental CA bundle was traced through the zona incerta into the dorsomedial part of the MFB in the middle hypothalamus. Here, it possibly joins locus fibres that have ascended within the CTT and through the tegmental radiations to reach the MFB at more caudal levels. Within the MFB the locus fibres then distribute to many diencephalic and telencephalic areas, as illustrated diagrammatically in Fig. 15. In agreement with previous fluorescence histochemical and autoradiographic studies 27, 42, 27, 28, 29 the major areas of termination of the system in the di- and telencephalon were found to be thalamus, neocortex, and hippocampus. The ascending locus axons were seen to project also to the tectum, pretectum, metathalamus, amygdaloid-piriform region, and probably to the septum and the olfactory bulb. These observations made in the intact animal have partly been confirmed in animals subjected to a bilateral destruction of the locus coeruleus (ref. 37 unpublished observations). At variance with the observations of Loizou 29 and Ungerstedt 24 however no significant termination of locus fibres could be established in the hypothalamus.

The rich collateralization of the locus coeruleus axons was directly observed in the GA-treated material, confirming the previous presumptions made on indirect evidence by Olson and Fuxe 24 and Ungerstedt 24. Thus, for example the DTB axons were seen to give off collateral branches towards the tegmental, tectal, and metathalamic regions, and further rostrally into the supraoptic decussations (towards metathalamic and pretectal areas) and the reticular and anterior thalamic nuclei (along the stria medullaris). Remaining axons then swept caudally in the cingulum to terminate in cortical and hippocampal areas. This branches

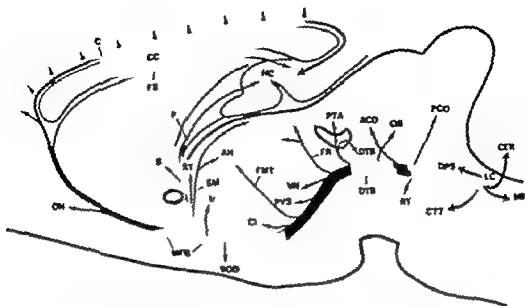


Fig. 15 Schematic representation of the course and primary branchings of the dorsal tegmental bundle originating in the CA cell bodies of the principal locus coeruleus. Compare with Fig. 1 and text. For abbreviations, see Index.

pattern suggests that one and the same locus neuron could influence — through collateral branches — several different regions in the brain. It is perhaps of particular interest that the widespread projections of locus coeruleus axons to both the specific thalamic nuclei and the neocortex provide a morphological basis for a representation of the activity of these neurons on both the cortical and the thalamic levels. In this context it should be noted that the rich collateralization is not unique for the locus coeruleus CA neurons, but a prominent feature also of the ascending projections from other pontine and medullary CA cell groups.

The ascending projections from the disseminated pontine and groups — together with fibres from the locus coeruleus — form an arranged fibre system in the CTT. It corresponds to the system as defined by Ungerstedt ⁴¹ and Olson and ⁴² and is a component of the ascending bundles within this present material — is illustrated diagram of origin of this system are situated in the A1 A5 cell groups of Dahlström and Fuxe ¹⁹. It is also contributed to the CTT system also from the extension of the coeruleus-subcoeruleus cerebellar peduncle into the roof of Such an arrangement would agree with retrograde changes in the A4 cells cell group. In agreement with the

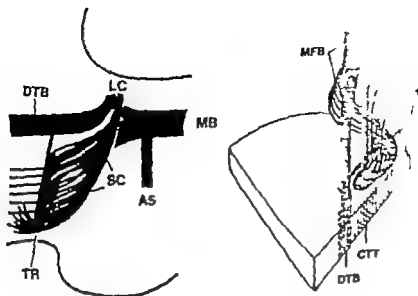


Fig. 16. The course of the CA fibre systems of the central tegmental tract through the so-called tegmental CA radiations in the mesencephalon as represented schematically in a lateral view (left) and in a dorsal view (right). Compare with Figs. 2 and 7 and text.

medullary portion of this system was observed to run dorsomedially in the CTT just ventrolateral to the DTB.

The CTT contributes importantly to the formation of the so-called tegmental CA radiations, from which the CA systems assemble at the meso-diencephalic junction to form part of the ascending MFB system (Fig. 16). The complex fibre arrangement in this region of the mesencephalic tegmentum, situated just rostral to the decussation of the superior cerebellar peduncle, consists partly of radiating preterminal axon bundles — following the characteristic fibre architecture of this part of the tegmentum ²² — and partly of densely arranged varicose terminal axonal ramifications. The dense CA terminal arrangement of this region has previously been described as the "nebula-like" formation by Maeda *et al.* ⁴¹ in the cat brain. The principal courses of different components of the CTT in this region are schematically illustrated in Fig. 16. Rostral to the tegmental radiations there are at least three different components of the CTT taking different projection routes. A ventral component, partly intermingled with the axons from the mesencephalic A8 cell group, runs just dorsal to the substantia nigra to join the MFB system in the region medial to the substantia nigra, and along the MFB the CTT fibres reach hypothalamic, thalamic, preoptic, septal, amygdaloid, and piriform regions. A lateral component passes along the optic tract within the SOD; a rostral component runs through the zona incerta and Rorel's Hs-field along a course corresponding to the so-called incerto-teme-tal and incerto-tectal tracts (cf. refs. 13, 25, 33). This latter component runs broadly into the internal capsule towards the amygdaloid-piriform region, the neostriatum, and possibly also into the neocortex, in addition fibres pass

observed to run into the SOD. The distributional pattern of the MFB component of the non locus CTT fibres conforms to that ascribed to the so-called ventral CA bundle by Ungerstedt ⁴¹ and Olsson and Fuxe ⁴² and to the so-called intermediate and ventral bundle by Maeda *et al.* ^{41, 42}. In addition, Maeda *et al.* ⁴¹ concluded from lesion experiments in the cat that the so-called intermediate pathway of the CTT would give terminals also to the occipital cortex, and Richardson and Jacobowitz ²⁹ and Sachs *et al.* ³⁷ in rats and mice treated with 6-hydroxydopa, observed presumed NA axons running through the internal capsule and the neostriatum into the overlying neocortex. In line with these observations, both the DTB and the zona incerta component of the CTT gave off fibres that ran broadly into the internal capsule, partly intermingled with DA fibres of the nigrostriatal pathway. These axons could partly be traced into the neostriatum (probably giving rise to the coarse varicose type of fluorescent terminal found scattered in this area), but in the GA treated material, the number of fibres that ran through the external capsule into the neocortex, having a fluorescence morphology of NA-containing axons of the locus or non-locus types, were sparse, although such fibres were observed. Thus, in the rat, the GA method does not provide support for a substantial neocortical NA innervation via the internal capsule and the neostriatum.

An important decussation of fibres of both the locus and the non-locus types was revealed within the *supraoptic decussations* (SOD): this agrees with the earlier findings of Maeda *et al.* ⁴¹ in the cat. The CA fibres ran into the SOD along three different routes: a) Caudally, in the rostral mesencephalon and the caudal diencephalon, fibres in the lateral part of the CTT curved around or through the crus cerebri towards the medial surface of the optic tract, i.e. along a course similar to that of the (partly myelinated) fibres of the ventral SOD ^{11, 45}. These lateral CTT fibres could therefore be regarded as an adrenergic component of this decussation. b) In the rostral hypothalamus, collateral branches from fibres of the locus and non-locus types left the MFB system ventromedially into the ventral and dorsal SOD. These fibres gave off abundant collaterals innervating large areas of the anterior and preoptic hypothalamic regions, including the supraoptic nuclei (see Figs. 12 and 13). The decussating fibres continue along the dorsomedial surface of the optic tract to project along the ansa lenticularis towards the amygdaloid-piriform region; others might continue further caudally along the optic tract towards the metathalamic and pretectal regions. c) Fibres leave the DTB and the CTT in the subthalamic region and run ventrally and laterally through the internal capsule into the SOD. Within the SOD, the CA fibres from the various sources intermingled, thus it was not possible to determine, in the normal intact animal, the exact course and termination of the different components of the decussating CA fibres. This obviously requires further experimental studies.

With the Marchi and Nauta stains of anterograde degeneration, Bucher and Bürgi ¹¹, Zyo *et al.* ⁴⁶ and Minderhoud ⁴⁵ have demonstrated fibres in the dorsal SOD that originate in the pontine reticular formation and ascend along the medial longitudinal fasciculus and through the zona incerta to decussate

the retrochiasmatic region. Zyo *et al* ⁶⁹ also traced fibres, after lesions in the midbrain and pontine tegmentum, along the ventrolateral part of the MFB into the ventral SOD. These systems, which are at least partly myelinated, are in all probability non-adrenergic. The course of the decussating adrenergic DFB and CTT fibres appears to be parallel to the decussating non-adrenergic systems and the adrenergic and non-adrenergic systems seem to intermingle in the rostral hypothalamus. From the observations in the quoted studies ^{11-42, 69} it seems in fact possible that the adrenergic and non-adrenergic components of the ventral and dorsal SOD to some extent have similar projections.

DOPAMINE-CONTAINING SYSTEMS

The mesencephalic CA cell groups — groups A 8, A 9 and A 10 of Dahlström and Fuxe ¹⁹ — are known to give rise to major ascending projections to the neostriatum and the limbic forebrain, and most of these neurons, at least, are known to be DA-containing ^{1, 3, 19-64}. Of these neuron systems, the nigrostriatal pathway — arising in cell bodies located in the substantia nigra (mainly within the pars compacta) and presumably also in the ventral tegmental area and in the A 8 cell group — has been established with a large variety of techniques, including fluorescence histochemistry, biochemistry, stains of terminal degeneration, and electron microscopy (for review of the literature see Moore *et al* ⁴⁷ and Carpenter and Peter ¹⁴). In addition, studies with the Falck Hillarp technique in conjunction with lesions have provided evidence for a projection of the mesencephalic DA cell groups (primarily the A 10 group) along the MFB to the nucleus accumbens, the olfactory tubercle and the interstitial nucleus of the stria terminalis ¹⁻⁶⁴. Ungerstedt ⁶¹ named these latter projections the mesolimbic DA system.

The characteristic fluorescence morphology of the presumed DA-containing axons in the GA-treated material made possible their distinction already in the normal untreated animal. In agreement with previous observations, the ascending DA fibres of the nigrostriatal and the mesolimbic pathways could thus be traced from the mesencephalic DA cell groups all the way up to the neostriatum and to the accumbens, olfactory tubercle and the interstitial nucleus of the stria terminalis respectively. Also previously unknown presumed DA-containing pathways were traced from the region of the mesencephalic cell groups to the septum, the frontal and anterior limbic cortices, the olfactory bulb and — via the ansa lenticularis — to the amygdala and the piriform cortex, the GA method thus disclosing far more wide-spread telencephalic projections of the mesencephalic DA cell groups than were previously known. The organization of the nigrostriatal, mesolimbic and mesocortical DA systems is schematically represented in Figs. 17 and 18.

The fibres assemble ventromedially at the meso-diencephalic junction and ascend as rather well-defined bundle, situated within and immediately lateral to the MFB. The different components within this system have an interesting topographic arrangement. The fibres going to the striatum and

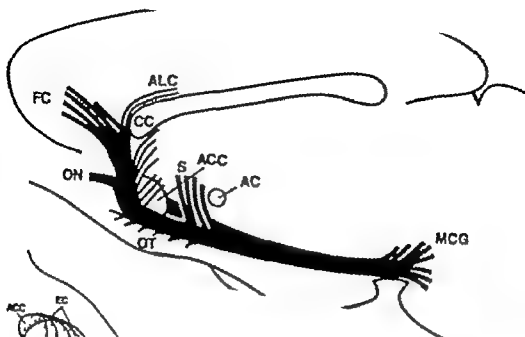


Fig. 17. Schematic representation of the presumed dopaminergic mesolimbic and mesocortical systems in a sagittal projection. For abbreviations, see Index.



Fig. 18. Schematic representation in a horizontal plane of the presumed dopaminergic projections from the mesencephalic CA cell groups (MCG) to the nucleus caudatus-putamen (NCP). Compare with Figs. 2, 10, and 12, and text. For abbreviations, see Index.

occupy the most dorsolateral part, then come the fibres to the interstitial nucleus of the stria terminalis, the fibres to the accumbens, the fibres to septum and the cortical areas, and most ventrally the fibres to the olfactory tubercle. Also within the nigrostriatal pathway there appeared to be at least a crude topographic arrangement, in that the fibres going to the caudal regions lie more dorsally and laterally and those going to the rostral and ventral regions lie more ventrally. This probably reflects the topographical relation demonstrated between the cells of the pars compacta of the substantia nigra and the neostriatum 2, 14. ■

Recently Thierry *et al.* 22, 23 presented biochemical evidence of a dopaminergic innervation of the neocortex, this has subsequently been supported by fluorescence histochemical and pharmacological observations 20, 24. In a parallel study using the OA method combined with stereotaxic lesions and CA determinations,

Lidvall *et al.* ²⁸ and Björklund *et al.* ⁸ demonstrated abundant DA-containing terminal systems in the frontal and the anterior limbic cortices which are the terminal projections of the cortical component of the ascending mesencephalic DA system described in the present paper. According to the lesion experiments, the fibres innervating the frontal cortex originate in the A 10 cell group and those innervating the anterior limbic cortex in the lateral part of the substantia nigra (i.e. the A 9 cell group). In contrast to the previously established noradrenergic neocortical innervation originating in the locus coeruleus ^{22, 24} these mesocortical dopaminergic pathways seem to have restricted areas of projection in the neocortex and their terminal density is considerably higher. Obviously the discovery of the extensive telencephalic projections of the mesencephalic dopaminergic neurons introduces entirely new aspects on the role of DA in the brain, these pathways providing alternative routes for brain stem influences on the neocortical functions.

GENERAL CONSIDERATIONS

The controversy that has existed regarding the projections of the monoamine neuron systems in the brain stem (see Moore ⁴⁶) resulted from the failure to trace with neuroanatomical techniques many of the telencephalic pathways that have been inferred from fluorescence histochemical and biochemical data. With respect to the nigrostriatal pathway the controversy was settled through the application of the more sensitive Fink Heimer and Watanen techniques for degenerating terminals, which allowed the direct tracing of this pathway ^{11, 42, 48}. Subsequently the nigrostriatal DA pathway has also been traced with the Falck Hillarp method in newborn mice ²⁴ and in human fetuses ⁴³. However also the more sensitive neuroanatomical staining techniques have failed to demonstrate significant ascending projections from the brain stem reticular forebrain structures ^{18, 40, 51, 52, 53} although there is substantial evidence of such projections of both CA-producing and indolamine-producing neurons in the brain stem (see refs. 1, 9, 41, 57, 59, 64 and others). The direct demonstration of the CA pathways to telencephalon with the GA method in the present study bridges this gap and supports the presumption that at least some of these fibre systems are too fine to be detected with the anterograde staining technique or are refractory to such staining.

The organization of the ascending CA neuron systems as revealed by the GA fluorescence method is highly complex. It reflects both the abundance of the CA fibre systems and their often complicated and interwoven courses, as well as their intricate suborganization and branching patterns, as well as the central CA neurons as highly diffuse and unspecific. The common notion of true only in a limited sense. Thus the ascending DA neuron systems appear to have topographically highly ordered projections, and the present observations indicate that also the ascending NA fibre systems have a high degree of suborganization. This, of course has special implications in experimental

involving lesions or stimulations of CA cell groups or fibre pathways. Thus, for example it is obvious from the organization of the projections from the locus coeruleus cell group that lesions at various points along the DTB — e.g. in the locus itself in the DTB behind the tegmental radiations, in the DTB in front of the tegmental radiations, in its course through the MFB or in the cingulum — will have different denervating effects and might thus have quite different functional consequences. Also it is evident from the present observations that lesions of the so-called ventral CA bundle of Ungerstedt¹¹ in the rostral mesencephalon (i.e. in the region of the tegmental radiations) or of the MFB system will involve several ascending CA fibre systems and will result in quite complex denervations. To overcome this and to provide possibilities for more precise experimental approaches to the different CA neuron systems, more work is necessary to clarify the suborganization of the CA pathways with respect to the origins and sites of termination of their fibres.

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**ROLE OF EN
HIST**
A study on

by
Curt

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**ROLE OF ENERGY METABOLISM IN
HISTAMINE RELEASE**

A study on isolated rat mast cells

by
Curt Peterson

 Stockholm, 1974

The present survey is based on studies carried out at the Department of Pharmacology Karolinska Institutet Stockholm. Apart from some hitherto unpublished results the studies have been presented in the following papers

- I Diamant B and C Peterson The metabolism of mono saccharides in isolated rat mast cells and its influence on histamine release induced by adenosine 5 triphosphate Acta physiol scand 1971 83 324-334
- II Peterson C and B Diamant Inhibitory action of exogenous adenosine 5 triphosphate on glycolysis in isolated rat mast cells Significance for histamine release Acta pharmacol et toxicol 1974 34 Preprint
- III Peterson C Inhibitory action of antimycin A on histamine release from isolated rat mast cells Acta pharmacol et toxicol 1974 34 Preprint
- IV Peterson C Histamine release induced by compound 48/80 from isolated rat mast cells Dependence on endogenous ATP Acta pharmacol et toxicol 1974 34 Preprint
- V Peterson C and B Diamant Increased utilization of endogenous ATP in isolated rat mast cells during histamine release induced by compound 48/80 Acta pharmacol et toxicol 1974 34 Preprint

These papers will be referred to by their Roman numerals

INTRODUCTION

The observation that antigen caused release of histamine from sensitized animals or tissues (Dragstedt and Gebauer Fuelnegg 1932 Bartosch Feldberg and Nagel 1932) suggested that histamine release is of importance in the pathogenesis of hypersensitivity reactions. Evidence for a role of energy metabolism in histamine release was first presented by Parrot (1942) who found that anoxia inhibited anaphylactic histamine release in guinea pig tissues. Combined biochemical and morphological studies indicated that histamine is present in high concentration in mast cells (Riley and West 1953 Fawcett 1954). This was confirmed when mast cells from the peritoneal cavity of the rat were concentrated by differential centrifugation (Padawa and Gordon 1955 Archer 1958). Fractionation of homogenized tissues demonstrated that histamine is stored in metachromatic granules (Mota et al 1954 West 1955).

A number of substances of relatively simple chemical structure have been found to cause extrusion of granules and histamine release from mast cells (see Paton 1957 Rothschild 1966). The histamine releasing property of compound 48/80, a condensation product of p-methoxyphenethylmethylamine and formaldehyde (Baltzly et al 1949) was originally demonstrated by Paton (1951). In the rat the histamine release process evoked by compound 48/80 has many characteristics (dependence on pH, temperature etc.) in common with anaphylactic histamine release (Högborg and Uvnäs 1960 Mota and Ishii 1960). In glucose free media the granule extrusion and histamine release were abolished by metabolic inhibitors (Junqueira and Beiguelman 1955 Högborg and Uvnäs 1957 1960 Mota and Ishii 1960). Histamine release induced by these agents was not accompanied by a loss of cytoplasmatic constituents (Diamant 1967a Johnson and Moran 1969). On the other hand substances such as long chain alkylamines and chlorpromazine caused degranulation of mast cells and histamine release.

which was not inhibited by metabolic inhibitors (Mota 1959 Högberg and Uvnäs 1960 Frisk Holmberg 1971) These agents also caused considerable loss of potassium and lactic dehydrogenase indicating a loss of cell integrity (Paton 1956 Frisk Holmberg 1971)

Glucose was found to counteract the inhibitory action of anoxia cyanide or dinitrophenol on histamine release from rat tissues induced by compound 48/80 or antigen antibody reaction (Diamant and Uvnäs 1961 Provoust Danon and Moussatché 1961 Rothschild Vugman and Rocha e Silva 1961 Diamant 1962b) This effect of glucose could be abolished by phlorizin an inhibitor of glucose transport (Diamant 1962a) From these and other studies it has been concluded that histamine release from rat mast cells induced by compound 48/80 or antigen antibody reaction is an energy dependent reaction which can be supported by oxidative or glycolytic energy yielding reactions

In view of the indirect evidence for the involvement of cellular ATP in the histamine release induced by compound 48/80 or antigen antibody reaction the effect of exogenous ATP on isolated rat peritoneal mast cells has been studied (Diamant and Krüger 1967 Sugiyama and Yamasaki 1969) It was found that ATP induced histamine release provided calcium was present However the histamine releasing ability of exogenous ATP does not seem to be due to its possible role as an energy donor since a number of inhibitors of cellular energy production were found to block the release (Diamant and Krüger 1967 Sugiyama and Yamasaki 1969 Diamant and Peterson 1970 Sugiyama 1971a) Glucose did not restore histamine release induced by exogenous ATP under anaerobic conditions (Diamant and Krüger 1967) However in the absence of metabolic inhibitors glucose enhanced histamine release induced by exogenous ATP but not by compound 48/80 (Diamant and Peterson 1970)

The present studies were undertaken in order to investigate the role of energy metabolism in histamine release from rat mast cells. The aims were

- 1 To compare the histamine release processes induced by exogenous ATP and compound 48/80 with regard to energy dependence
- 2 To correlate the ability of the mast cells to release histamine when exposed to compound 48/80 with the content and turnover of endogenous ATP in the cell
- 3 To study the energy metabolism in the mast cell histamine release to establish whether or not the energy dependence reflects an increased utilization of endogenous ATP

METHODS

The methods used have been described in detail in the individual papers and therefore only a summary will be given below

1 Isolation of rat mast cells

Mast cells were isolated from the peritoneal and pleural cavities of male Sprague Dawley rats following essentially the method of Uvnäs and Thon (1959-1961, Thon and Uvnäs 1966). The cell suspension obtained contained 90-95% mast cells. In each experiment at least two different aliquots were counted in a Bürker chamber to determine the concentration of mast cells (500-2000 cells counted).

2 Analytical procedures

Histamine was assayed fluorometrically after condensation with phthalaldehyde (OPT) as described by Shore, Burkhalter and Cohn (1959). The extraction procedure was omitted since histamine was the only substance present in rat mast cells which gave fluorescence in the presence of

OPT (Bergendorff and Uvnäs 1972) Histamine release has been expressed as a percentage of the total histamine content of the cells

Analyses of ATP glucose and lactate were performed according to the methods developed by Lowry and co workers (see Lowry and Passonneau 1972) These methods are based on measurements of the native fluorescence of the reduced forms of the pyridine nucleotides In the presence of the appropriate enzyme(s) the particular substrate is oxidized by NAD or NADP (or reduced by NADH or NADPH) whereby the pyridine nucleotide itself is reduced (or oxidized) When ATP was determined the fluorescence of NADPH was increased by oxidation in a strongly alkaline medium Creatine phosphate was determined as ATP after the addition of creatine kinase Glycogen was determined as glucose after boiling the samples in HCl The fluorescence of NADH or NADPH (or histamine OPT) was determined in a Farrand Ratio Fluorometer with appropriate filters for measuring the fluorescence at 460 nm after excitation at 366 nm The assays were based on two readings made before and after the addition of the samples (for determination of ATP or lactate) or an auxiliary enzyme (hexokinase for determination of glucose) to the reagent solution in the fluorometer tubes The concentration of ATP glucose or lactate in the cell samples was determined by comparison with identically treated standards In some experiments internal standards were also run

The concentration of ATP creatine phosphate glucose glycogen or lactate in the cell samples has been expressed as moles/kg mast cells (dry weight) The dry weight of the mast cells was determined from the cell count and the mean dry weight of single peritoneal mast cells (0.5 ng/cell Diamant and Lowry 1966 Diamant and Glick 1967) The ATP content (per cell) in the contaminating cells was about half of that in the mast cells (Johnson and Moran 1969) The lactate concentration in suspensions of mixed peritoneal cells was less than 1/3 of that in suspensions of isolated mast cells (same total cell concentration) before

incubation as well as after incubation with glucose and antimycin A. Since the cell suspension contained 90-95% mast cells, no correction had to be made for the contribution from contaminating cells.

3 Statistical methods

Mean values and standard errors of the mean obtained in experimental series of at least 4 experiments of 3 experiments ranges have been given. The statistical significance of differences between means was tested with the Student's t test. The level of significance is denoted by p values.

RESULTS AND DISCUSSION

1 Energy metabolism in isolated mast cells

a) Endogenous substrates

Mast cells seem to have a good supply of endogenous substrates. Thus, during incubation at 37° in a glucose free medium, the oxygen consumption remained unchanged for at least 4 hours (Chakravarty and Zeuthen 1965) and the ATP content was not reduced within 1 hour (Diamant 1967b). The lactate content in the cell suspensions was 5.0 ± 0.46 mmoles/kg dry weight (mean value ± S.E.M., n = 33) immediately after isolation and it did not change during incubation in a glucose free medium at 37° for 15 min (I-IV). The glycogen content immediately after isolation was about 5 mmoles of glycosyl units/kg dry weight.

Only traces of glucose and glucose 6 phosphate were found. From the oxygen consumption data given by Chakravarty and Zeuthen (1965) it can be calculated that the glycogen present would be consumed in about 1 hour if the cells only utilized glycogen. This suggests that the mast cells also utilize other endogenous substrates such as lipids.

b) Phosphagens

The ATP content in suspensions of untreated mast cells amounted to 1.59 ± 0.08 μ moles/kg dry weight (mean value \pm S.E.M. n = 31) (III-IV-V). This value agrees well with those found by Diamant (1967b) and Bergquist, Samuelsson and Uvnäs (1972) but is somewhat less than the values reported by Johnson and Moran (1969) and Johansen and Chakravarty (1972). The mast cells contained much less creatine phosphate than ATP (Fig. 1) which is quite the reverse of what has been found in muscle tissue (see Karlsson 1971). As a rule only ATP was measured.

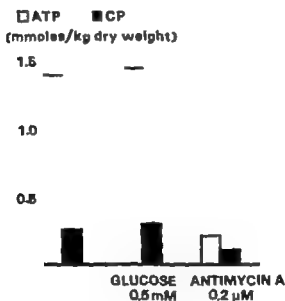


Fig. 1

Contents of ATP and creatine phosphate (CP) in rat mast cells. The cells were incubated for 6 min (at 37°) in the presence or absence of glucose (0.5 mM) or antimycin A (0.2 μ M). Mean values from 2 experiments are shown.

c) Metabolism of glucose

The addition of glucose to suspensions of mast cells caused an accumulation of lactate which was related to the glucose concentration up to about 0.5 mM (I-IV). Using ^{14}C glucose Ho Cabut and Meng (1970) found that the lactate formation increased with the glucose concentration above 1 mM. The discrepancy might be ascribed to the longer incubation periods used by these authors (12 hours compared to 15 min in the present studies). The accumulation of lactate was linear with time for at least 30 min (II) and the maximal rate under aerobic conditions was about $1 \text{ mmol} \times \text{kg dry weight}^{-1} \times \text{min}^{-1}$. This is in good agreement with the lactate production rate calculated indirectly by Chakravarty (1966) when mast cells were incubated in a bicarbonate medium containing 5 mM glucose. The accumulation of lactate accounted for 60-75% of the glucose utilization (IV) which is in good agreement with the results of Ho Cabut and Meng (1970).

Glucose (0.5 mM) caused a slight increase in the ATP content of the mast cells (IV). This can be attributed to the enhanced glycolytic flux and also to enhanced oxidative phosphorylation since glucose elevated the oxygen consumption of mast cells (Chakravarty and Zeuthen 1965). No additional rise in ATP content was observed when the glucose concentration was further increased.

d) Metabolism of other monosaccharides

Mannose (0.5 mM) was metabolized to lactate by the mast cells to the same extent as was glucose whereas no lactate accumulation was observed on incubation with fructose, galactose or L-glucose (0.5 mM of each) (I). Chakravarty (1968) found that in addition to glucose fructose and galactose (5 mM) were also oxidized by rat mast cells. However, no lactate accumulation occurred when the cells were incubated with 5 mM fructose or galactose even under anaerobic conditions. This indicates that fructose and galactose are not metabolized to a significant rate by the glycolytic pathway in rat mast cells. A similar

pattern of metabolism of monosaccharides has been found in the pancreatic islets of the rat (Jarret and Keen 1968) and the mouse (Hellerström 1967 Ashcroft Weerasinghe and Randle 1973)

e) Effect of metabolic inhibitors

Iodoacetate used as an inhibitor of glycolysis (Webb 1966) abolished the lactate accumulation from glucose but did not affect the lactate content in the absence of exogenous substrates (I)

Antimycin A which is known to inhibit electron transport in the mitochondrial respiratory chain (Wainio 1970 Slater 1973) caused lactate to accumulate from endogenous substrates (I). The accumulation rate was $0.17 \text{ } \mu\text{moles} \times \text{kg dry weight}^{-1} \times \text{min}^{-1}$. Antimycin A also enhanced lactate accumulation from glucose (I-III). The accumulation increased with the glucose concentration up to 1.1 mM (IV) at which concentration the accumulation rate amounted to about $3 \text{ } \mu\text{moles} \times \text{kg dry weight}^{-1} \times \text{min}^{-1}$. These results are at variance with those of Chakravarty (1965) who found no significant difference between the rates of anaerobic glycolysis in the presence and absence of glucose (5 mM). Our results agree with the view that the restoring effect of glucose on histamine release induced by compound 48/80 or antigen antibody reaction is due to an increased glycolytic energy yield (Diamant 1962b)

The utilization of glucose was increased by antimycin A (IV) confirming the suggestion of a Pasteur effect in the mast cells (Chakravarty 1965). In the presence of antimycin A lactate accumulation accounted for 82-97% of the glucose utilization (IV) as compared to 60-70% in its absence.

Antimycin A reduced the cellular ATP content to about 30% of the original value within 3 min (III) which is in good agreement with observations on the reduction of the ATP content in mouse pancreatic islets after the addition of an uncoupling agent (Ashcroft Weerasinghe and Randle

1973) The oxidative ATP generation in mast cells seems to be completely blocked by $0.2 \mu\text{M}$ antimycin A since pyruvate (4.7 mM) had no restoring effect on the ATP content (IV). In contrast glucose in only one tenth of the pyruvate concentration restored the ATP content to 70-80% of the original value. Furthermore antimycin A ($0.2 \mu\text{M}$) has been shown to abolish the $^{14}\text{CO}_2$ production from $2 \text{ }^{14}\text{C}$ pyruvate in mast cells (Diamant and Morn unpublished observation). It should be pointed out that $0.03 \mu\text{M}$ antimycin A has been found to completely inhibit the respiration of cultured lymphoma cells (Gregg Machinist and Currie 1968).

In the light of these observations it seems reasonable to assume that the rate of lactate accumulation in suspensions of mast cells in the presence of antimycin A ($0.2 \mu\text{M}$) gives a reliable measure of the cellular ATP production. From the rate of lactate accumulation after the addition of various concentrations of glucose to antimycin A treated cells it was calculated that the turnover time of ATP in the cells was about 30 sec irrespective of the steady state level (IV). This indicates that the rate of utilization of ATP in mast cells is directly proportional to the ATP content.

■ Energy dependence of histamine release induced by exogenous ATP

a) Inhibition of histamine release by antimycin A

Histamine release induced by exogenous ATP and compound 48/80 showed the same sensitivity towards the inhibitory action of antimycin A (III). It was not necessary to preincubate the mast cells with antimycin A to block ATP induced histamine release as was the case when compound 48/80 was used. This can be explained by the fact that histamine release caused by ATP is a relatively slow process with an initial lag period of about 1 min (Diamant and Peterson 1970 Sugiyama 1971a) whereas 48/80 induced histamine release is completed within 10 sec (Bloom Fred

holm and Haegermark 1967 Sugiyama 1971a) The length of the lag period was reduced by increasing the concentration of ATP (Dahlquist and Diamant 1974) This finding may explain the incomplete inhibition of histamine release observed when concentrated mast cell suspensions were exposed simultaneously to antimycin A and high concentrations of ATP (II)

b) Failure of glycolytic substrates to restore histamine release under anaerobic conditions

Glucose did not restore ATP induced histamine release in the presence of inhibitors of oxidative metabolism (Diamant and Krüger 1967 I II) Mannose was also ineffective in that respect This finding can be explained by the observation that exogenous ATP but not compound 48/80 inhibited the glycolytic metabolism of glucose in rat mast cells (II) Similarly the metabolism of mannose was inhibited The mechanism behind this action is not known It could be attributed to an inhibition of some glycolytic reaction(s) or to a reduced uptake of monosaccharides

c) Enhancement of histamine release by glycolytic substrates under aerobic conditions

Under aerobic conditions glucose and mannose i.e. those monosaccharides which were metabolized to lactate in the mast cells enhanced histamine release induced by exogenous ATP but not by compound 48/80 (Diamant and Peterson 1970 I) Lactate and pyruvate also enhanced the release (I) The enhancing effect of glucose but not that of lactate or pyruvate was abolished by 0.1 mM iodoacetate which caused a partial inhibition of the histamine release in the absence of exogenous substrates (Diamant and Peterson 1970 I) The results indicate that in the absence of exogenous substrates the energy production rate is a limiting factor for histamine release induced by exogenous ATP because of the inhibitory action of ATP on glycolysis in the mast cells However since the inhibition was not complete it seems likely that the addition of glucose

CU-1 is a small, light-colored, rectangular object, possibly a piece of wood or plastic, with a smooth surface and rounded edges. It is positioned horizontally in the center of the frame. The background is a plain, light-colored surface, possibly a wall or a backdrop, with some faint, vertical lines visible. The lighting is even, highlighting the object's shape and texture.

1. The first step is to identify the problem or goal. This involves understanding the current situation and what needs to be achieved. It is important to be clear and specific about the objectives.

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1. The first step is to identify the problem or question that needs to be answered. This involves understanding the context and the specific requirements of the task.

1. The purpose of this report is to provide a summary of the results of the study conducted by the research team. The study was designed to investigate the effects of the proposed intervention on the target population. The results indicate that the intervention had a significant positive impact on the outcome measures. The findings suggest that the intervention is effective in addressing the research objectives. The study was conducted in a controlled environment, and the results are consistent with previous research. The research team is confident in the validity of the findings. The study was completed on time and within budget. The results are presented in a clear and concise manner. The research team is grateful for the support and assistance provided by the funding agency. The study is a valuable contribution to the field of research. The findings are expected to inform future research and practice. The research team is committed to continuing its efforts to advance the field of research. The study is a testament to the dedication and hard work of the research team. The results are a testament to the effectiveness of the proposed intervention. The study is a testament to the importance of research in advancing knowledge and improving outcomes. The research team is proud of the results and is committed to sharing them with the research community. The study is a testament to the power of research to drive positive change. The research team is grateful for the opportunity to conduct this study and is committed to continuing its efforts to advance the field of research. The study is a testament to the importance of research in advancing knowledge and improving outcomes. The research team is proud of the results and is committed to sharing them with the research community. The study is a testament to the power of research to drive positive change.

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has also been found after treatment with oligomycin (Johansen and Chakravarty 1972)

It should be noted that a correlation has been found between the ATP content and the rate of glucose induced insulin release from mouse pancreatic islets after the addition of various metabolic inhibitors (Ashcroft Weerasinghe and Randle 1973)

b) Restoring effect of glycolytic substrates on histamine release from antinycin A treated cells

Monosaccharides (glucose or mannose) which were metabolized to lactate by the mast cells restored the ability of antinycin A treated mast cells to release histamine on exposure to compound 48/80 (IV). The restoring effect occurred concomitantly with an increase in the cellular ATP content which attained a steady state level within 2.5 min. Histamine release was completely restored by 0.51 mM glucose which gave rise to a steady state level of ATP of about 75% of the original value.

Conclusion The ability of rat mast cells to release histamine when exposed to compound 48/80 is correlated to their ATP content (Fig. 2). Practically no histamine can be released from cells in which the ATP content has been reduced to about 25% of the original value. On the other hand an ATP content of about 75% of the original level is sufficient to give maximal histamine release.

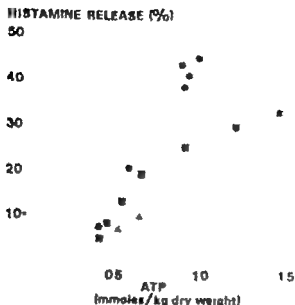


Fig 2

Histamine release induced by compound 48/80 and its relation to the ATP content in the mast cells. The cells were treated for different times with antimycin A or glycolase and antimycin A before the addition of compound 48/80 (Results from Fig 3 in paper III (squares), fig 4 (circles) and fig 5 (triangles) in paper IV).

4 Utilization of endogenous ATP during histamine release

From observations on the effects of metabolic inhibitors and substrates on histamine release induced by compound 48/80 or antigen antibody reaction it has been suggested that the release process involves utilization of cellular ATP (Moussatché and Provoust-Danon 1958, Chakravarty 1962, Diamant 1962b, Rothschild 1963, Kahl and Matter 1967). However, Diamant (1967b) found no decrease in the ATP content of rat mast cells 15 sec - 60 min after the addition of compound 48/80.

In view of the rapid turnover of ATP in rat mast cells the question of whether or not histamine release is accompanied by an increased utilization of ATP was reinvestigated using cells in which the production of ATP has been reduced by pre incubation for 1 min with antimycin A (V). In such cells histamine release induced by compound 48/80 was accompanied by a reduction in ATP content which exceeded that in cells not exposed to the releasing agent ($p < 0.005$). The observation by Diamant (1967b) that histamine release induced by compound 48/80 from cells with an intact energy production was not accompanied by a decrease in cellular ATP content was confirmed. The results contradict the hypothesis presented by Yamazaki (1967) and supported by Sugiyama (1971b) that energy is not needed for the histamine release process per se. According to their proposal an active energy metabolism in the mast cells is a prerequisite for the integrity and function of the cells including the ability to respond to a histamine releasing agent such as compound 48/80. A similar proposal has been made for the secretion of vasopressin from the neurohypophysis (Thorn 1970).

Histamine release induced by chlorpromazine or *n*-decylamine was not accompanied by a decrease in cellular ATP regardless of whether antimycin A was present or not. However, on prolonged incubation with these agents the ATP content decreased. This might be explained by a release of cytoplasmic constituents and subsequent hydrolysis of the ATP released and/or by a reduced production of ATP in the cells.

Conclusion The results indicate that histamine release induced by compound 48/80 is accompanied by an increased utilization of endogenous ATP in the mast cells. This escaped detection when cells with intact metabolic pathways were used, presumably because of the rapid ATP production in such cells.

5 Comparison of histamine other secretory systems

Histamine release from rat m 48/80 or antigen antibody re in common with other secretor tion of catecholamines from cn medulla insulin secretion fro and vasopressin secretion from posterior lobe of the hypophysi that the secretory products are s rounded granules and morphologica evidence have been presented whic cretion occurs by exocytosis of the n Douglas 1968)

Histamine seems to be released from the mas i o ules as the result of a cation exchange between hi tar e ionically bound to a protein heparin complex the granules and extracellular cations (Uvnäs 1964 Uvnäs and Thon 1966 Uvnäs Aborg and Bergendorff 1970) However the percentage of histamine (or serotonin) released has been reported to exceed the percentage of granules extruded (Carlsson and Ritzén 1969 Mosál Slorach and Uvnäs 1970 Slo ach 1971) This can be explained by the observation that some granules are retained within cellular cavities in communication with the extracellular medium (Röhlich Anderson and Uvnäs 1971 Lagunoff 1972 Anderson Slorach and Uvnäs 1973) Although the initial course of events is believed to involve fusion of the perigranular membrane with the cell membrane (Röhlich Anderson and Uvnäs 1971 Anderson Slorach and Uvnäs 1973 Lagunoff 1973) the proposed mechanism for histamine release seems to obviate a need for transport of all granules to the cell surface

Based on similarities between the excitation contraction coupling in muscle tissue (Sandow 1952) and the secretory process the term stimulus secretion coupling has been suggested for the sequence of events triggered by a secretagogue (Douglas and Rubin 1967) In addition to endogenous

ATP calcium is believed to have an important function in this process (see Rubin 1970a). However omission of calcium in the medium did not abolish the effect of compound 48/80 on isolated rat mast cells (Uvnäs and Thon 1961 Van Arsdale and Bray 1961 Sasaki 1964 Diamant and Krüger 1967) whereas degranulation and histamine release from tissue mast cells were blocked (Högborg and Uvnäs 1960 Hata and Ishii 1960 Sasaki 1964). The possibility remains however that intracellular depots of calcium are of importance for histamine release from isolated mast cells induced by compound 48/80. This view is supported by the finding of Douglas and Ueda (1973) that prolonged preincubation of mast cells in the presence of EDTA abolished the release and that it was restored by the introduction of calcium.

Recently it has been demonstrated that the addition of an ionophore for calcium caused histamine release and degranulation of mast cells in a calcium containing medium (Foreman Monger and Gomperts 1973 Cochrane and Douglas 1974). The release was accompanied by an uptake of calcium and could be inhibited by metabolic inhibitors (Foreman Monger and Gomperts 1973). Degranulation of mast cells has also been found after intracellular injection of calcium by some authors (Kanno Cochrane and Douglas 1973) but not by others (Tasaka et al 1970).

A relationship between calcium and cyclic AMP as intracellular messengers in the stimulus secretion coupling has been suggested (Rasmussen 1970). In contrast to most other systems histamine release from rat mast cells induced by compound 48/80 was inhibited by dibutyryl cyclic AMP or agents supposed to raise the cyclic AMP level (Loeffler Lovenberg and Sjoerdsma 1971). However no correlation has so far been established between the increase in cyclic AMP content in isolated rat mast cells and the inhibition of the histamine release (Johnson Moran and Mayer 1973).

The concept that secretion is an energy dependent process is principally based on observations on the blocking action of metabolic inhibitors (cf Hokin 1951 Coore and Randle 1964 Douglas Ishida and Polsner 1965 Kirshner and Smith 1966 Rubin 1969 1970b Ball et al 1969) However some doubts have been raised concerning the direct involvement of energy metabolism (Thorn 1970)

It has been suggested that the secretion of granular material from blood platelets induced by thrombin or collagen is accompanied by an increased utilization of endogenous ATP since the decrease in intracellular ATP content exceeded the amount of ATP which was recovered extracellularly (Holmsen Day and Storm 1969 Day and Holmsen 1971) However it has been questioned if the breakdown of intracellular ATP during the secretion is connected with the secretory process (Ireland 1967) The observation that catecholamine secretion from the adrenal medulla under certain conditions was accompanied by glycogen depletion (Rubin 1969) gives indirect support for the concept that energy is utilized during secretion

One difficulty concerned with studies on adrenal medulla and blood platelets is that most cellular ATP is stored in the amine containing granules and metabolically inactive (Falck Hillarp and Högberg 1956 Prusoff et al 1961 Stjärne 1964 Holmsen Day and Storm 1969) The observation that antimycin A did not completely deplete the ATP content in mast cells (III IV) might indicate that mast cells contain a small pool of metabolically inactive ATP However the mast cells granules only contained traces of ATP (Bergquist Samuelsson and Uvnäs 1971) and Johnson and Moran (1969) did not find any release of ATP during histamine release induced by compound 48/80 or antigen antibody reaction

It is not known which reaction(s) in the secretory process are dependent on energy One possibility is that energy is utilized by a contractile process It has been suggested

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ACTA PHYSIOLOGICA SCANDINAVICA
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Cardiorespiratory
and Metabolic Functions
During Exercise
in the Hyperbaric Environment

BY

LENNART FAGRAEUS

STOCKHOLM 1974

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The present publication is based on studies reported in the following papers

- I. Fagraceus L., C. M. Hesser and D. Linnarsson. Cardiorespiratory responses to graded exercise at increased ambient air pressure. *Acta physiol. scand.* In press.
- II. Fagraceus L., J. Karlsson H. Linnarsson and B. Saltin. Oxygen uptake during maximal work at lowered and raised ambient air pressures. *Acta physiol. scand.* 1973 87 411-421.
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In the text these papers will be referred to by their Roman numerals I - V

PREFACE

The present investigation was performed at the Departments of Aviation and Naval Medicine Karolinska Institutet Stockholm

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Lennart Fagraeus

Symbols and abbreviations

The symbols and abbreviations in this investigation conform to the guidelines devised by the Glossary Committee of the International Union of Physiological Sciences (see Glossary Committee of the IUPS 1973 a, b)

Primary symbols

P	Partial pressure of gas given in mm Hg at actual body temperature
V	Gas volume in general
V	Gas volume per unit time
F	Fractional concentration in dry gas phase
ATA	Atmosphere(s) absolute. 1.0 ATA is equivalent to 760 mm Hg or $1.013 \cdot 10^5 \text{ N m}^{-2}$ or 1.013 bar or to the pressure exerted by approx. 10.0 meters of sea water

Suffixes

I	Inspired gas
E	Expired gas
T	Tidal gas
A	Alveolar gas
D	Dead space

Other symbols

STPD	Standard temperature and pressure dry (0°C , 760 mm Hg)
ATPS	Ambient temperature and pressure saturated with water vapor
BTPS	Body temperature, barometric pressure and saturated with water vapor
W	Watt. $1 \text{ W} = 6 \text{ L} \cdot \text{lpm} \cdot \text{min}^{-1}$

Symbols used for the statistical treatment of data

n	Number of subjects or observations
Range	Smallest and greatest value observed
M	Arithmetic mean
SD	Standard deviation
SEM	Standard error of the mean
p	Probability

INTRODUCTION

It is known since ancient times that man is capable of living and working in the high-pressure environment. This environment involves changes in a number of physical factors such as partial pressures of O_2 and N_2 , gas density and pressure per se, which may exert definite physiological effects. The foundation of our knowledge of these effects was laid by Paul Bert (1878). He and later Boycott, Damant and Haldane (1908) recognized that there is danger not only in remaining at pressure but also in decompression to the normal environment. It is now well established that the increased N_2 pressure during air breathing in deep diving exerts a narcotic influence which affects psychomotor performance (for review see Bennett 1968) and that increased air density increases the airway resistance with a consequent reduction of the ventilatory capacity (for review see Lauphler 1968). High oxygen pressures may exert toxic effects on the cells of the central nervous system and on the respiratory epithelium (for review see Lambertsen 1965). Finally it is known that high pressures per se can influence biological processes although it is still not possible to identify the exact point where pressure exerts the first possible effect (for review see Fenn 1969). To avoid the adverse effects of air at high pressures, gas mixtures of helium and low concentrations of oxygen have been used in diving to great depths.

Comparatively little is known about the physiological adjustments to muscular exercise that occur in the high-pressure environment. Triger (1841) and Friedberg (1872) seem to have been the first to report physiological effects of muscular work at high air pressures (cited by Hoff 1948). Triger, who used a caisson designed by him for a working depth of 20 meters, i.e. for a pressure of 3.0 atmospheres absolute (ATA), reported that laborers claimed that when ascending the ladder from the working chamber while still under pressure they felt much less out of breath than when performing similar exertion

in the open air Friedberg in reviewing the historical development of compressed air work, stated that there was acceleration of the metabolic processes and that workers claimed that they had greater muscular power while subjected to high pressure

In spite of the fact that air has so far been and probably always will be the breathing gas of choice in caisson work and in diving operations to moderate depths, relatively few investigations have been undertaken to study the physiological adjustments to muscular exercise during acute exposure to hyperbaric air Ventilatory responses to submaximal exercise at increased air pressures have been studied by La Lanphier (1963) Jarrett (1966) Taunton et al (1970) Miller Wagonsteen and Lanphier (1971 1972) Wood and Bryan (1971) Lally Moore and Hong (1971) and Broussolle et al (1972) There is general agreement that ventilation becomes depressed when the air pressure is raised above normal and also that this depression at least in part is related to the increase in gas density Secondary to the inadequate ventilatory response to exercise in hyperbaric air alveolar PCO_2 may rise considerably This increase in alveolar PCO_2 is more marked in trained divers than in subjects who have not previously been exposed to raised air pressures (Lanphier 1963 Jarrett 1966 Broussolle et al 1972) The oxygen uptake ($\dot{V}O_2$) during heavy exercise at 2.0 ATA air was measured in two subjects by Taunton et al (1970) who observed an average increase in $\dot{V}O_2$ of about 14 % as compared to 1.0 ATA air This increase in $\dot{V}O_2$ was ascribed in part to the increased work of breathing a denser gas and in part to an increased efficiency of oxygen utilization at 2.0 ATA air On the other hand Lally et al (1971) observed no increase in $\dot{V}O_2$ during light to moderate exercise at 2.0 ATA air Heart rate during light exercise in hyperbaric air was studied by Shilling Hawkins and Hansen (1936) who found a progressive decrease in exercise heart rate when the ambient air pressure was raised stepwise to 10 ATA They attributed this relative bradycardia to the increase in O_2 pressure

Little is known about the physiology of maximal exercise in the hyperbaric environment At the moderately raised air pressure of 1.14 ATA

(Wyndham et al 1970) and 1.19 ATA (Eagan and Plesse 1969) maximal oxygen uptake has been found to be significantly increased this change being ascribed by Wyndham et al to the increase in oxygen pressure. However when the oxygen pressure was raised to 3.0 ATA Kaijser (1970) observed no changes in maximal oxygen uptake and performance time. When the inspired oxygen pressure was kept constant a rise in the N_2 pressure up to approximately 3.0 ATA reduced the maximal oxygen uptake by 32 %, and ventilation by 62 % (Cook 1970). At 3.0 ATA air Varène, Jacquemin and L'Huillier (1972) found no consistent change in either oxygen uptake or ventilation during exhaustive exercise as compared to 1.0 ATA air. However these authors reported that four out of five subjects were able to exercise at a higher work load in the hyperbaric condition. Whereas Eagan and Plesse (1969) reported that maximal heart rate was lower than control at 1.19 ATA air. Wyndham et al. (1970) and Varène et al (1972) found no significant changes in heart rate at exhaustion at 1.14 and 3.0 ATA air respectively.

The objectives of the present investigation were to study the effects of acute exposure to raised ambient air pressures on the adaptation of cardio-respiratory and metabolic functions to graded submaximal and maximal exercise and furthermore to evaluate the separate effects of increases in O_2 pressure, N_2 pressure, gas density and pressure per se. For this purpose exercise data obtained on subjects breathing air at raised pressures in the range 1.0 - 3.0 ATA were compared to those obtained on the same subjects breathing oxygen at 1.0 and 1.3 ATA or a mixture of 21 % O_2 in helium at 3.0 ATA.

METHODS AND PROCEDURES

Descriptions of the methods and procedures used in this investigation have been given in detail in papers I - V and are therefore only briefly summarized below. The experimental conditions to which the subjects were exposed in the separate studies are presented in Table I.

Subjects

The experiments were performed on 21 healthy volunteers, 10 of which participated in more than one study. They were all physically active and some were well-trained in endurance sports. All subjects were familiar with exposure to raised ambient pressures; seven were professional Navy divers. Those subjects who participated in the maximal exercise tests were all well acquainted with the sensations experienced in exhaustive exercise. Anthropometric and functional data for the group of subjects are summarized in Table II. Individual data having been reported in the respective papers.

Compression chamber and breathing system

All experiments were performed in a dry compression chamber with an inner diameter of 2.1 m, length 3.9 m, volume 1.6 m³. The temperature in the chamber could be set at predetermined levels by means of a radiator-fan assembly controlled by a thermostat (Billman, Sweden). In all experiments the chamber temperature was kept at 21-23°C and the pressure controlled to within 1 mm Hg of any given preset level. The experimental procedure inside the chamber was closely supervised through port holes in the chamber wall and by means of closed-circuit TV combined with an inter-communication system.

The subjects were provided with gas mixtures of known and constant composition from high pressure tanks outside the chamber. Inside the chamber the gas from the high pressure tank was allowed to pass either a 200 liter

TABLE I Experimental conditions in studies I V

Study	n	Inspired gas	Ambient Pressure ATA	Work Load
I	7	Air	1 0 4 5	50 100 150 W
		O	1 0	
II	7	Air	1 0 1 13 1 40	50-60 of $\dot{V}O_{2\max}$ and max exercise
			2 0 3 0	
III	6	Air	1 0 1 40	50 of $\dot{V}O_{2\max}$ and max exercise
IV	8	Air	1 0 3 0 6 0	100 W and max exercise
		21.4 % O_2 in helium	3 0	
V	6	Air	1 0 6 0	50 W
		O_2	1 3	

TABLE II Anthropometric and functional data of 21 subjects

	Age yrs	Height cm	Weight kg	$\dot{V}O_{2\max}$ *) l min ⁻¹ STPD
Mean	28.8	180.9	73.2	3.74
SD	4.2	6.1	6.8	0.71
Range	23-36	171-189	56-81	2.16-5.40

*) In seven subjects (I) $\dot{V}O_{2\max}$ was predicted according to Astrand (1960)

Douglas bag partially filled with water or a large water bottle to become humidified and then via wide bore tubing was led past the inspiratory side of a low resistance breathing valve (dead space 10 ml) (v. Döbeln 1919) at a flow rate of about 3.5 l min^{-1} ATPS. In this way since the volume of the wide bore tubing distal to the point of attachment of the breathing valve exceeded 1.5 liters no chamber air was inspired by the subject even during maximal inspiratory effort. In one study (I) the subject inhaled the humidified gas in the Douglas bag via a Venturi type low resistance flowmeter (Wigertz 1969). Expired gas was collected in 300 liter Douglas bags and the volumes were measured at atmospheric pressure by means of a balanced Tissot spirometer (II, III, IV). The chamber was continuously vented as needed to maintain constant inside pressure.

Exercise tests

All experimental data were obtained with the subjects in the sitting position at rest or performing submaximal or maximal exercise on a mechanically (I, II, III) or electrically (IV, V) braked cycle ergometer. The motor box of the latter ergometer (Elema Schönmänder, Sweden) was continuously and slowly flushed with 100 % nitrogen to exclude fire hazard from raised O_2 pressures in the hyperbaric experiments. The work loads used in the various studies (I - V) are shown in Table I. In the study on graded submaximal exercise (I) the subjects exercised for 6 min at each of three consecutive loads of 80, 100 and 150 W and in the study on autonomic blockade (V) 6 min periods of rest and exercise at 50 W were alternated repeatedly. In the studies on maximal exercise the maximal work tests were preceded by ≈ 5 min of 'warm-up' exercise at loads corresponding to 50-60 % of $\text{VO}_{2\text{max}}$ (II, III) or at 100 W (IV). The work loads in the maximal exercise tests were chosen on an individual basis so as to bring the subjects to exhaustion within 3-5 min under normobaric condition, breathing air. This supramaximal work level was determined on a separate day prior to the experiments and averaged for the group: 1.2 % of the maximal aerobic power of the subjects as determined at 1.0 ATA breathing air. By keeping the duration of the maximal exercise

tests within 3 \pm min any potential risk for decompression sickness in the hyperbaric experiments was minimized

Methods

In study I pulmonary ventilation was measured by means of a Venturi-type flowmeter in combination with on-line digital computation techniques (Åström and Wigertz 1966) yielding tidal volume (V_T) respiratory frequency (f) and inspired minute volume (V_I). In the other studies (II, III, IV) ventilation (V_E) was obtained from expired volumes collected in Douglas bags. The composition of inspired and expired gases was analyzed on duplicate samples according to Scholander (1947). Based on the above data, oxygen uptake (\dot{V}_{O_2}) carbon dioxide elimination (\dot{V}_{CO_2}) and ventilatory equivalents for O_2 (\dot{V}_E/\dot{V}_{O_2}) and carbon dioxide (\dot{V}_E/\dot{V}_{CO_2}) were obtained. For recording of end tidal P_{CO_2} gas from the mouthpiece (IV) or from a special breath-by-breath sampler (Brisman, Heeser and Matell 1962) (I) was led through the chamber wall and analyzed continuously for CO_2 by means of external fast responding analyzers (Capnograph Godart or Model LB-I, Beckman Instruments). In one study on maximal work (II) alveolar P_{CO_2} was calculated by Bohr's equation assuming the V_D/V_T ratio to be 0.12 in exhaustive exercise (cf. Asmussen and Nielsen 1966). All respiratory data in the submaximal exercise experiments were measured as time-averages over the 6th min of work at each level, whereas those on maximal exercise represent the highest mean values obtained over the last two 30 s intervals in exhaustive exercise.

Heart rate (HR) was recorded beat by-beat by means of chest electrodes and a linear cardiotelemeter (Lindborg, Wigertz and Ödman 1969). Submaximal HR values were obtained as time-averages over the 6th min of work at each load, whereas maximal HR (HR_{max}) was defined as the peak value at the point of exhaustion.

Blood lactate concentration was obtained from arterialized capillary samples from the finger tip analysed according to Barker and Summerson (1941) as modified by Ström (1949) (II, IV) or by an enzymatic method adapted for small blood samples (Scholtz *et al.* 1959) (III). Samples for determination

of peak blood lactate concentration were taken 1 and 4 min after cessation of the maximal work test.

Muscle metabolites were examined by taking specimens from the lateral portion of the thigh (vastus lateralis of the quadriceps femoris muscle) (III). The muscle samples were obtained by means of a needle biopsy technique (Borgström 1963). Skin and fascia were anaesthetized with 1-2 ml of Lidocain (1%) and a short incision (5-10 mm) was made with a scalpel. These preparations were made with the subject in the supine position. Muscle specimens were taken before and immediately after cessation of submaximal and maximal exercise. The specimens were frozen in liquid nitrogen within 3-4 s after the needle was withdrawn from the muscle and then stored at -80°C until analyzed for ATP, CP, glycogen, glucose, glucose-6-phosphate, pyruvate and lactate with methods described by Karlsson (1971).

Inspiratory gas flow \dot{V}_T , \dot{V}_I and tidal \dot{V}_{CO_2} and HR were recorded continuously and simultaneously on an 8-channel ink recorder (Brush Mark 200). A 14-channel analog (FM) magnetic tape recorder (Ampex FR 100 C) was used for storage and subsequent off line analysis of data.

Procedures

In all five studies (I-V) the experiments at raised pressures were carried out according to the same general procedure as used in the corresponding control situation at 1.0 ATA air, except for the compression and decompression phases. Following compression, at least 5 min was allowed for the subject to become adjusted to the conditions and for chamber temperature to attain equilibrium. In each experiment the subject in the chamber was accompanied by at least one assistant experimenter who carried out various tasks according to the experimental protocol in collaboration with the investigator outside the chamber. Decompression to atmospheric pressure was accomplished on air according to standard decompression tables. No symptoms of decompression sickness were observed. Before each experiment routine medical examinations were made to ensure that the subjects were in good physical condition.

Analysis of data

To estimate the separate effects of raised O_2 pressure data from the O_2 experiments at 1.0 and 1.3 ATA or from the He- O_2 experiments at 3.0 ATA were compared to those obtained on air at 1.0 ATA (I-IV-V). The effects of raised N_2 pressure were disclosed by comparing data from the air experiments at 4.5 and 6.0 ATA to those obtained on O_2 at 1.0 and 1.3 ATA respectively or by comparing the air data at 3.0 ATA to the He- O_2 data at the same pressure (I-IV-V). The combined effects of increased O_2 and N_2 pressures were obtained by comparing the air data at raised ambient pressures to those at 1.0 ATA (I-V).

Conventional statistical methods have been applied for the calculation of arithmetical mean, standard deviation and standard error of the mean. The statistical significance of differences between mean values were evaluated by applying the Student t-test to the intraindividual differences (of Fisher 1948).

RESULTS

The results are presented in two main sections dealing with the adaptation of cardiorespiratory and metabolic functions to exercise during acute exposure to hyperbaric environments with special emphasis on the separate effects of increases in inspired O_2 pressure, inert gas pressure, gas density and pressure per se. The first section deals with submaximal exercise performed at different preset work loads, whereas the second section presents data obtained during maximal exercise. For the sake of clarity only the main results pertaining to selected variables are included. For additional information the reader is referred to the detailed accounts given in the separate papers (I - V).

A. Submaximal work

The values given in this section refer to the stable-state situation (6th min) of exercise performed at ergometric work loads of 50, 100 and 150 W under three different ambient conditions: viz. with the subjects breathing air at 1.0 ATA, oxygen at 1.0 ATA and air at 4.5 ATA (I, Table III). In the case of the heart rate, additional values are included for submaximal exercise (50-60 % of $\dot{V}O_{2\max}$) at 1.13, 1.40, 2.0 and 3.0 ATA air (II) and for light exercise (50 W) at 1.3 ATA O_2 and 6.0 ATA air before and after autonomic blockade (V).

Oxygen uptake ($\dot{V}O_2$) increased in a linear fashion with the ergometric work load both at 1.0 and 4.5 ATA air (I, Fig. 1). At the highest load (150 W) $\dot{V}O_2$ was 12 % greater ($p < 0.01$) in the hyperbaric environment. The regression equations relating $\dot{V}O_2$ to work load (wl) in the two conditions were

$$\dot{V}O_2 = 486 + 10.07 \text{ wl} \quad \text{at 1.0 ATA} \quad (n = 21, r = 0.978)$$

$$\dot{V}O_2 = 464 + 11.67 \text{ wl} \quad \text{at 4.5 ATA} \quad (n = 21, r = 0.989)$$

where $\dot{V}O_2$ is expressed in $\text{ml} \cdot \text{min}^{-1}$ STPD and wl in watt.

In the O_2 experiments at 1.0 ATA the exercise V_{O_2} values were assumed to be similar to those observed at 1.0 ATA air as it has been shown that substituting oxygen for air at normal atmospheric pressure has no significant influence on V_{O_2} in submaximal exercise (Asmussen and Nielsen 1965).

Carbon dioxide elimination (V_{CO_2}) also increased linearly with the ergometric load in all three conditions. At all work loads V_{CO_2} was somewhat greater in the hyperbaric than in the two normobaric conditions. This increase in V_{CO_2} in the high pressure condition was proportional to the increase in V_{O_2} however as indicated by the fact that the respiratory exchange ratio did not change significantly when the air pressure was raised from 1.0 to 4.5 ATA.

When related to the ergometric work load the ventilatory response to graded exercise was similar in all three conditions except that the pulmonary ventilation (V_I) was significantly reduced by 9 % at the highest load when oxygen was substituted for air at 1.0 ATA. That a rise in the ambient air pressure to 4.5 ATA and thus in N_2 pressure to 3.5 ATA did not affect V_I at any given load (Fig. 1) would seem to indicate that high N_2 pressure had no significant influence on the ventilatory response to exercise. However since V_{O_2} and thus the metabolic load was greater in the hyperbaric environment it follows that V_I was less for a given metabolic load at 4.5 ATA breathing air than at 1.0 ATA breathing air or oxygen (Fig. 2). At a V_{O_2} of 2.0 l min⁻¹ the reduction in V_I due to the rise in N_2 pressure was calculated to average 3.3 l min⁻¹ or approximately 8 %. That the pulmonary ventilation was reduced both by high P_{O_2} and by high P_{N_2} is also reflected by the fact that the ventilatory equivalents for oxygen (\dot{V}_I/V_{O_2}) and for CO_2 (\dot{V}_I/V_{CO_2}) were reduced at 100 and 150 W when oxygen was substituted for air at 1.0 ATA and further reduced when the pressure during air breathing was raised to 4.5 ATA.

End-tidal P_{CO_2} increased with the work load in all three conditions (Fig. 4). Because of the reduction in ventilatory response to 150 W during O_2 breathing at 1.0 ATA end-tidal P_{CO_2} rose significantly from a control value of 45 to 48 mm Hg. The further non- O_2 -dependent reduction in V_I observed when the pressure was raised to 4.5 ATA caused significant

increases in end-tidal P_{CO_2} at all three work loads. At 150 W in the 4.5 ATA experiments end-tidal P_{CO_2} averaged 53 mm Hg.

Heart rate (HR) increased linearly with the work load in all three conditions (I). However, at a given ergometric load, HR decreased consistently as the ambient air pressure was raised (I, II, V). This lowering of the heart rate was found to be more marked when HR was related to a given \dot{V}_{O_2} (I). For any given \dot{V}_{O_2} , HR was lower during O_2 breathing at 1.0 ATA and still lower at 4.5 ATA breathing air than in the control situation (I, Fig. 5). The regression equations relating HR to \dot{V}_{O_2} were:

$$HR = 48.2 + 40 \cdot \dot{V}_{O_2} \quad \text{at 1.0 ATA air} \quad (n = 8, r = 0.923)$$

$$HR = 47.6 + 37.0 \dot{V}_{O_2} \quad \text{at 1.0 ATA } O_2 \quad (n = 28, r = 0.884)$$

$$HR = 30.2 + 36.6 \dot{V}_{O_2} \quad \text{at 4.5 ATA air} \quad (n = 28, r = 0.945)$$

where HR is expressed in beats min^{-1} and \dot{V}_{O_2} in $\text{l} \cdot \text{min}^{-1}$ STPD.

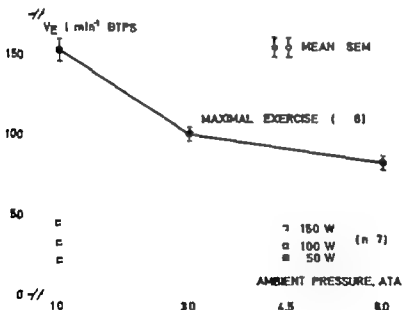


Fig. 1. Steady state ventilation during submaximal (50, 100 and 150 W) exercise at 1.0 and 4.5 ATA air and ventilation during the last 30 s of exhaustive exercise at 1.0, 3.0 and 6.0 ATA air (based on data from I and IV).

The contributions of sympathetic and parasympathetic influences to the O_2 - and M_2 induced decreases in HR were studied by means of differentiated autonomic blockade induced by administration of propranolol (10 mg i v) or atropine (2 - 2.5 mg i v) or both drugs in combination (V) In experiments with light exercise (50 W) at 1.3 ATA O_2 and 6.0 ATA air ($PO_2 = 1.3$ ATA) it was found that the O_2 -induced reduction in HR was unaffected after propranolol and somewhat less after atropine alone or in combination with propranolol The non- O_2 -dependent decrease in HR on the other hand remained unaffected after atropine but was significantly reduced after propranolol alone or in combination with atropine

II Maximal work

In this section respiratory heart rate and blood lactate data refer to maximal exercise breathing air in the pressure range 1.0 - 6.0 ATA (II - III IV) and He- O_2 at 3.0 ATA (IV) Data on muscle metabolites were obtained at 1.0 and 1.40 ATA air (III) All data refer to mean values obtained over the last 30 s of exhaustive work unless otherwise stated

Oxygen uptake ($V_{O_2\max}$ Fig. 2) increased by 3 and 8.11 % respectively when the ambient pressure was increased to 1.13 and 1.40 ATA (II - III) However with a further rise in pressure to 2.0 and 3.0 ATA $V_{O_2\max}$ was reduced below values at 1.40 ATA (II) and at 6.0 ATA was not significantly different from control (IV) With He- O_2 at 3.0 ATA $V_{O_2\max}$ was increased by 11 % above control values at 1.0 ATA air (IV)

Carbon dioxide elimination increased slightly as the ambient pressure was raised to 1.40 ATA and then decreased when the pressure was further raised to 2.0 ATA (II) At 3.0 ATA V_{CO_2} was actually lower than at 1.0 ATA air (II - IV) At 6.0 ATA V_{CO_2} was significantly lowered by 0.49 l min^{-1} STPD below control value (IV) Inhalation of He- O_2 at 3.0 ATA however caused no consistent change in V_{CO_2} (IV)

Pulmonary ventilation (Figs. 1 and 2) decreased as the ambient air pressure was raised At 1.40 ATA this decrease averaged 7 % (II) at 3.0

ATA 33 % (II IV) and at 6.0 ATA 46 % (IV). With He-O_2 at 3.0 ATA ventilation was decreased by 14 % as compared to control values at 1.0 ATA air (IV).

End-tidal P_{CO_2} (Fig. 2) which averaged 33.3 mm Hg at 1.0 ATA air rose with the ambient pressure. At 1.40 ATA the increase averaged 10 % and at 2.0 and 3.0 ATA 21 and 39 % respectively (II IV). At 6.0 ATA the increase amounted to no less than 63 % as compared to control (IV). At 3.0 ATA He-O_2 end-tidal P_{CO_2} was 13 % higher than at 1.0 ATA air (IV).

Heart rate at the point of exhaustion (HR_{max} Fig. 3) amounted to 185.7 beats min^{-1} at 1.0 ATA and showed no consistent differences in

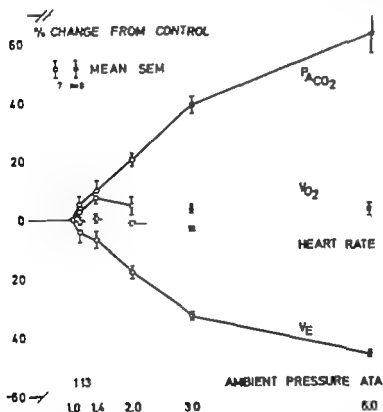


Fig. 2 Cardiorespiratory adjustments in maximal exercise at increased atmospheric air pressures. Data compiled from II and IV and shown on a percentile basis, values at 1.0 ATA air being taken as 100 % (P_{ACO_2} values calculated or uncorrected and tidal values).

the pressure range up to 2.0 ATA (II). At 3.0 and 6.0 ATA, however, HR_{max} was significantly decreased by 3 and 5 % respectively as compared to control (II-IV). With 3.0 ATA $He-O_2$, HR_{max} was significantly lower than at 1.0 ATA air but higher than at 3.0 ATA air (IV).

Peak blood lactate concentrations did not differ significantly between the various pressure levels (1.0 - 6.0 ATA) (II-III-IV). Oxygen deficit and concentrations of muscle lactate, ATP, CP, pyruvate, glucose and glucose-6-phosphate as well as glycogen depletion at exhaustion remained unchanged when the air pressure was raised to 1.40 ATA (III).

Endurance time, i.e. the time from start of maximal exercise to exhaustion, was related to the ambient pressure in a manner similar to $\dot{V}O_{2max}$. Thus, endurance time was significantly prolonged by 15.0 % at 1.40 ATA (II-III) but showed no further increase when the pressure was raised to 2.0 and 3.0 ATA (II). At 6.0 ATA air, endurance time did not differ from control, whereas at 3.0 ATA $He-O_2$ it was significantly prolonged by 14 % (IV).

DISCUSSION

Ventilation and CO_2 elimination

It is known that the ventilatory capacity is reduced in the hyperbaric air environment (for review see Lamphier 1969) and also that exercise ventilation is significantly decreased (Lamphier 1963 1967 1969 Jarrett 1966 Taunton *et al* 1970 Lally Moore and Hong 1971 Miller Wangensteen and Lamphier 1971 1972 Wood and Bryan 1971 Broussolle *et al* 1972). However no attempts have so far been made to evaluate the relative contributions of raised PO_2 and PN_2 to the reduction of the ventilatory response to exercise in hyperbaric air.

In the present investigation the separate effect of raised O_2 pressure on ventilation (\dot{V}_I) during graded submaximal exercise was studied by substituting O_2 for air at 1.0 ATA (1). At the highest work load (160 W) \dot{V}_I decreased significantly by approximately 9 %, but showed no consistent changes at 80 and 100 W. That \dot{V}_I was reduced by hyperoxia in heavy but not in mild exercise is in agreement with earlier reports (cf. Asmussen and Nielsen 1946 1958 Bannister and Cunningham 1954 Lambertsen *et al* 1959). This effect of hyperoxia has been ascribed to improved aerobic conditions in the working muscles and to withdrawal of the hypoxic ventilatory drive originating in the arterial chemoreceptors (cf. Asmussen and Nielsen 1958). To evaluate the effect of raised N_2 pressure the ventilatory data from the 4.5 ATA air experiments were compared to those obtained at 1.0 ATA O_2 (1). When related to the metabolic (\dot{V}_{O_2}) rather than the ergometric load \dot{V}_I was found to be reduced by about 8 % at an O_2 uptake of $2.0 \text{ l} \cdot \text{min}^{-1}$ STPD as the inspired N_2 pressure was raised to 3.5 ATA (total air pressure = 4.5 ATA). Thus at this metabolic load the shares contributed by O_2 - and non- O_2 -dependent factors to the total ventilatory reduction were of similar magnitude at 4.5 ATA air.

That the non- O_2 -dependent reduction in ventilation was due to a narcotic effect of the increased N_2 pressure seems unlikely in view of previous studies on breath-holding (Hessler 1962 1965) and on ventilatory responses to CO_2 (Fagraeus and Hessler 1970 Lambertsen et al 1973) in hyperbaric air which indicate that high N_2 pressures (up to at least 6 # ATA) have no significant narcotic influence upon neural structures involved in the control of respiration. Other studies have shown, on the other hand that the ventilatory response to exercise (Tabakin and Hanson 1960 Cerretelli Sikand and Farhi 1969) and CO_2 (Cherniack and Siskind 1956 Milic Emili and Tyler 1963) are diminished when the work of breathing is increased at 1.0 ATA by addition of an external resistance. It may be concluded therefore that the non- O_2 -dependent reduction of V_I observed at 4.5 ATA air was due mainly to increased airway resistance caused by the increased gas density.

The separate effect of high O_2 pressure on ventilation during maximal exercise ($V_{E\max}$) was studied by comparing data obtained with He- O_2 at 3.0 ATA to those with air at 1.0 ATA (IV). The threefold increase in PO_2 in the presence of normal gas density in the He- O_2 experiments resulted in a significant reduction of $V_{E\max}$ (14 %) which is analogous to that observed with O_2 -enriched gas mixtures at normal atmospheric pressure (cf. Asmussen and Nielsen 1946 1958 Bannister and Cunningham 1954 Deroanne et al 1973). The further 20 % reduction in $V_{E\max}$ observed when air was substituted for He- O_2 at 3.0 ATA provides a measure of the non- O_2 -dependent decrease of $V_{E\max}$ during air breathing at this pressure (IV). Since the O_2 -dependent decrease of $\dot{V}_{E\max}$ at normal atmospheric pressure has been found to be maximal already at an inspired O_2 concentration of 66 % (Bannister and Cunningham 1954) equivalent to the O_2 pressure in the present 3.0 ATA air experiments. It can be assumed that the O_2 -induced reduction of $V_{E\max}$ at 6.0 ATA was of similar magnitude as at 3.0 ATA. The 16 % reduction in $V_{E\max}$ observed at 6.0 ATA air would thus be predominantly due to non- O_2 -dependent factors.

The importance of increased gas density in reducing the ventilatory capacity has been investigated by J. A. Maio and Farhi (1967) who found

gradual decrement in maximum voluntary ventilation with increased relative gas density no matter whether this was achieved by using a dense gas at normal atmospheric pressure by raising the ambient pressure or by a combination of both. Increased gas density augments the airway resistance and thereby the work of breathing which has been suggested as a limiting factor for heavy exercise at depth (cf. Lamphier 1967). Recent findings have shown however that the power dissipated by the ventilatory muscles during exhaustive exercise remains small as compared to the total work performed also when the ambient air pressure is raised to 3.0 ATA (Vardas, Jacquemin and L'Huillier 1972). An important consequence of increased gas density at high ventilatory rates is the flow limitation that occurs in the airways due to dynamic compression (for reviews see Lamphier 1969, Wood and Bryan 1971). It may therefore be surmised that the non- O_2 -dependent reduction of $\dot{V}_{E\max}$ observed at raised air pressures in the present experiments was largely due to such density induced flow limitation.

As a result of the reduced ventilatory responses to submaximal and maximal exercise in the present hyperbaric air experiments the CO_2 elimination was impeded with a consequent marked rise in end-tidal P_{CO_2} . Thus the relative hyperventilation with regard to metabolically produced CO_2 that is normally induced in exhaustive exercise (Asmussen 1965) was prevented at 3.0 and 4.0 ATA which must have resulted in a severe respiratory acidosis superimposed on the metabolic acidosis produced by lactate formation (II, IV). Also the CO_2 tension in the blood is raised and the CO_2 transport from the tissues impeded by hypoxia if the latter is great enough to cause an increase in the oxygen saturation of the venous blood (cf. Lambertsen 1965).

Oxygen uptake

During submaximal exercise in air \dot{V}_{O_2} at a given ergometric load was found to be about 10 % higher at 4.5 ATA than at 1.0 ATA (I). This rise in \dot{V}_{O_2} can apparently not be ascribed to the increase in O_2 pressure since it has been shown that a similar rise in O_2 pressure during comparable work loads at normal ambient pressure exerts no significant influence on \dot{V}_{O_2} (Asmussen and Nielsen 1955). Similarly it seems unlikely that the high N_2

pressure or the pressure per se would contribute to the increased \dot{V}_{O_2} at 4 ATA as it has been shown that the oxygen consumption rate of isolated tissues such as the rat diaphragm or brain tissue remained unchanged when the P_{O_2} pressure was raised to 4.7 ATA at an O_2 pressure of 1.0 (Severinghaus and Roughton 1946; Rodgers, Fenn and Craig 1969). It seems reasonable to conclude therefore that the higher than normal \dot{V}_{O_2} at 4.5 ATA air was caused by the increased work of breathing induced by the increase in gas density and hence in airway resistance. Further support for this conclusion can be obtained from studies by Glauser. Glauser and Rusy (1967) and Salzano, Rabin and Saltzman (1970). In experiments on the ventilatory response to CO_2 at normal atmospheric pressure Glauser et al. found that the O_2 cost of breathing increased significantly when the density of the inspired gas was increased by substituting SF_6 for nitrogen in the inhaled CO_2 air mixture. Salzano et al. observed in exercise experiments that the \dot{V}_{O_2} for a given ergometric load was significantly higher at 31.3 ATA breathing a mixture of helium-oxygen the density of which was 4.4 times that of air at 1.0 ATA. In both investigations the extra \dot{V}_{O_2} found during inhalation of the denser gases was ascribed in large part or entirely to the increased work of breathing.

During maximal exercise \dot{V}_{O_2} max was significantly increased by 21% at 1.4 ATA air (II, III). This effect may be ascribed to the rise in P_{O_2} pressure since it is well established that \dot{V}_{O_2} max increases significantly when extra oxygen is offered to the working muscles e.g. by breathing O_2 -enriched gases at normal atmospheric pressure (Ullrich, Long and Lupton 1934; Nielsen and Hansen 1937; Margaria et al. 1961, 1970; Dorenbosch et al. 1973). That the increased work of breathing due to increased gas density at 1.4 ATA air contributed to the rise in \dot{V}_{O_2} max seems unlikely as it has been shown that an increase in the external airway resistance and hence in work of breathing does not cause any significant increase of \dot{V}_{O_2} max at 1.0 ATA air (Correia, Silland and Farhi 1969; Damedtz and Anthonsen 1973). When the ambient air pressure was raised to 2.0 and 3.0 ATA (II) no further increase in \dot{V}_{O_2} max was observed in spite of O_2 pressures which under normal atmospheric conditions would yield clearcut increases in \dot{V}_{O_2} max. On the contrary

at 2.0 and 3.0 ATA air \dot{V}_{O_2} max was lower than at 1.4 ATA and at 6.0 ATA air it was not significantly different from control. The observation that with He-O₂ breathing at 3.0 ATA \dot{V}_{O_2} max increased by no less than 0.46 l min⁻¹ whereas with air breathing at the same pressure no distinct change in \dot{V}_{O_2} max occurred (IV) indicates that factors related to the increased N₂ pressure opposed the beneficial influence of the high O₂ pressure on \dot{V}_{O_2} max. The clue to the unchanged \dot{V}_{O_2} max at 3.0 and 6.0 ATA air should be sought in the defective CO₂ elimination in hyperbaric air secondary to increased gas density and hypoventilation (cf. above). For as shown in recent experiments at normal barometric pressure breathing through increased resistance (Cerretelli, Sikand and Farhi 1969; Hermansen, Vokac and Loroim 1972; Demedts and Anthonisen 1973) or the addition of 15 mm Hg P_{CO₂} to the inspired air (Laft Finkelstein and Elliott 1974) decreases the maximal O₂ uptake attainable during exercise. A similar mechanism was presumably involved in the hyperbaric air experiments where the reduced ventilatory response to exercise led to marked hypercapnia. With the data at hand however it cannot be settled whether this "CO₂ effect" noted by setting a limit due to respiratory distress, or by interfering with the supply or utilization of oxygen in the working muscles. Also it cannot be excluded that N₂ molecules interfered with the diffusion of O₂ in the maximal exercise experiments in which metabolic rates were greatly increased.

Heart rate

The results obtained during submaximal exercise in hyperbaric air (I, II, V) confirm the old observation by Shilling, Hawkins and Hansen (1935) that high pressures of air exert a depressant effect on heart rate, an effect that these authors attributed to the elevated O₂ pressure. The present data (I, V) clearly demonstrate however that the relative bradycardia observed at 4.5 and 6.0 ATA air could only in part be explained by the high P_{O₂}, the remaining non-O₂-dependent part being caused by the increased P_{N₂}, gas density or ambient pressure per se acting either alone or in combination. It is well established that hyperoxia causes a decrease in heart rate at

submaximal work, both when induced at normal (Assmussen and Nielsen 195) and at high ambient pressures (Salzano et al 1967 Kaijser 1970 Taunton et al. 1970) This effect has been attributed to an increase in vagal tone since in the resting condition, it is prevented by administration of atropine (Daly and Bondurant 1962) Results from the present study (V) indicate however that during light exercise the major part of the O_2 -dependent reduction of heart rate can be ascribed to a direct influence of hyperoxia on the heart whereas the remaining part is due to an increased parasympathetic tone

A non- O_2 -dependent slowing of heart rate in submaximal exercise has recently been reported by Flynn Berghage and Coll (1972) to occur in humans breathing normoxic gas mixtures of varying density at constant or varying ambient pressures A similar effect has also been obtained during exercise in helium atmospheres at pressures up to and including 31.3 ATA (Hamilton 1967 Salzano Ramsch and Salzman 1970 Bradley et al 1971 Schaefer Carey and Dougherty 1971) Flynn et al. (1972) obtained evidence that this relative bradycardia was due to the combined effects of increments in ambient pressure and gas density although no explanation for the underlying mechanism was offered. In the isolated guinea-pig heart preparation Fagraceus (1971) on the other hand was unable to detect any effects of raising the N_2 pressure to 8.0 ATA or the hydrostatic pressure to 9.0 ATA on heart rate myocardial contractile force or the response to sympathetic stimulation. These results together with the findings in a recent study by Fagraceus Haggendal and Linnarsson (1973) that the mean arterial pressure and the noradrenaline levels in arterial blood during heavy exercise at 6.0 ATA air showed no consistent changes in the face of a lowered heart rate seem to indicate that the non- O_2 -dependent reduction in heart rate is due to a subnormal response to sympathetic stimulation. This concept is supported by the present observation that the non- O_2 -dependent slowing of the heart was significantly less after beta adrenergic blockade (V)

Data about heart rate during maximal exercise in hyperbaric air are lacking in the literature In the present investigation the reduction in heart

rate was smaller during maximal than during submaximal exercise at air pressures up to and including 3.0 ATA (II). A possible explanation for this discrepancy in the heart rate responses may be that HR_{max} is not influenced by O_2 pressures up to 1.0 ATA (cf. Miller et al. 1953; Margaria et al. 1973; Deroanne et al. 1973). Thus the observed decreases in HR_{max} in the present investigation were apparently of non- O_2 -dependent origin (II-IV). The observation of a reduced HR_{max} in the He- O_2 experiments at 3.0 ATA (IV) indicates an effect on the heart either by the helium gas itself or by the pressure *per se*. The further reduction in HR_{max} induced by substituting air for He- O_2 at 3.0 ATA (IV) was apparently caused either by the increased gas density or the raised N_2 pressure. The additional reduction of HR_{max} in the 6.0 ATA air condition (IV) may be similarly explained as caused by the increase in gas density, N_2 pressure or pressure *per se* or by a combination of these factors. Although the true nature of this non- O_2 -dependent reduction of HR_{max} is not known, it seems likely that it can be attributed to a subnormal response of the heart to adrenergic stimulation in conformity with the situation in submaximal exercise discussed above (cf. V. Fagardus, Högendal and Linnarsson 1973). Since the cardiac stroke volume during maximal exercise has been shown to be unaffected both by an increase of the inspired O_2 pressure to 0.5 ATA (Ekblom 1974) or 3.0 ATA (Kajiser 1970) and by beta-receptor blockade (Epstein et al. 1968), it seems probable that the changes of HR_{max} in the present study do reflect corresponding changes of cardiac output, i.e. reductions of cardiac output of only about 2% in the He- O_2 experiments at 3.0 ATA and 4-5% in the air experiments at 3.0 and 6.0 ATA (IV).

Point of exhaustion

It has been the objective of many studies to find a causal connection between a biochemical or a physiological variable on the one hand and the subjective perception of exhaustion on the other. A major problem is that several factors contribute to setting the individual perception of the point of exhaustion, depending *i.e.* on the type of maximal exercise test that is performed. Psychological factors such as motivation, familiarity with the

different experimental conditions and tolerance to discomfort and pain experienced in exhaustive exercise are important. In the present investigation all subjects were well acquainted with both the maximal exercise procedure and with exposure to raised ambient air pressures. Thus any influence of psychological factors on the point of exhaustion was minimized except possibly in the 6.0 ATA air experiments where respiratory distress and dizziness were present in varying degrees. Among physiological factors a depletion of the glycogen store in the muscle cell seems unlikely as a limiting factor since all maximal exercise tests were of relatively short duration (cf. Saltin and Karlsson 1971). Increased acidity in the muscle cell is known to decrease enzyme activity (cf. Danforth 1965) and a decreased pH in the muscle (Hajler 1970, Hermansen and Osnes 1972) or in the blood (Cerretelli 1967) has been proposed as a limiting factor for exhaustive exercise of short duration. Accumulation of lactate with a consequent increased acidity in the muscle cell has recently been suggested by Hultman and Bergström (1973) to be a major factor in bringing about muscular fatigue either through inhibition of glycolysis or by preventing the rate of glycolysis to increase during the terminal part of exercise.

In the present investigation peak blood lactate and muscle lactate at exhaustion remained unchanged no matter whether maximal exercise was performed in normoxia or hyperoxia or whether hypoxemia or marked hypercapnia was present (II, III, IV). This observation strongly favors the concept that maximal work performance is more closely related to the lactate concentration than to the P_{CO_2} or pH levels in the blood or in the working muscles at the point of exhaustion. It would thus seem that maximal work is limited by a critical level of lactate concentration possibly by inducing a product inhibition of lactic dehydrogenase (LDH) a hypothesis recently suggested by Karlsson, Hultén and Sjödin (1974) based on an *in vitro* study of human skeletal muscle LDH.

The shorter endurance time in the 3.0 and 6.0 ATA air experiments compared to that observed at 3.5 ATA $He-O_2$ would therefore indicate that

the rate of lactate accumulation was higher in the former conditions although the inspired O_2 pressure was the same or higher. The explanation for this increase in the rate of lactate accumulation may be sought in the same mechanisms as were responsible for the failure of V_{O_2} max to increase in the 3 \bar{P} and 6 \bar{P} ATA air experiments (cf. above)

GENERAL SUMMARY

The bulk of physiological research on muscular exercise has so far been restricted mainly to the normal atmospheric environment. To investigate the effects of acute exposure to the hyperbaric environment on physiological functions in submaximal and maximal exercise 21 healthy subjects were exposed to increased ambient pressures in the range 1.0 - 6.0 ATA. The main objective of this investigation has been to evaluate the separate and combined effects of high O_2 and N_2 pressures on cardiorespiratory and metabolic functions. This was done by comparing exercise data obtained under different ambient conditions i.e. with the subjects breathing air, O_2 or He- O_2 at various elevated pressures (cf. Table I p. 11).

A. Submaximal work

1. Oxygen uptake and carbon dioxide elimination increased linearly with the work load both at 1.0 and 4.5 ATA air. Both variables showed a tendency to be higher in the hyperbaric condition and at 160 W $\dot{V}O_2$ was increased by 12% above control. There were no gross differences between the increases in $\dot{V}O_2$ and $\dot{V}CO_2$ as reflected by an unchanged respiratory exchange ratio when the air pressure was raised to 4.5 ATA.

2. Whereas pulmonary ventilation at 160 W decreased significantly by 9% when the O_2 pressure was increased by substituting O_2 for air at 1.0 ATA no reduction of ventilation was observed at 4.5 ATA air as compared to control in spite of the raised O_2 pressure (1.0 ATA). Since $\dot{V}O_2$ was increased above control at 4.5 ATA air it follows however that ventilation at a given $\dot{V}O_2$ was lower in the hyperbaric condition. At a $\dot{V}O_2$ of 2.0 l min⁻¹ STPD the total ventilatory reduction at 4.5 ATA amounted to approximately 17% as compared to 1.0 ATA air, about half of which could be ascribed to the O_2 -dependent decrease in ventilation. The remaining part

1.0 the non- O_2 -dependent reduction of ventilation could be attributed mainly to the higher gas density causing an increase in airway resistance and hence in the work of breathing

3 As a consequence of the reduction in ventilation at 4.5 ATA air end-tidal PCO_2 was significantly raised at all three work levels (50, 100 and 150 W). During 150 W at 4.5 ATA end-tidal PCO_2 was 18 % higher than control whereas the corresponding value at 1.0 ATA O_2 was 7 % above control reflecting the O_2 -dependent decrease in ventilation.

4 Heart rate was significantly decreased at all work levels during exposure to 4.5 ATA, the decreases being more evident when related to V_{O_2} . The sub-normal heart rate response with hyperbaric air could only in part be ascribed to the raised O_2 pressure, the remaining part being caused by some factor related to the increase in N_2 pressure. By the use of differentiated autonomic blockade evidence was obtained that the O_2 -dependent decrease in heart rate was due both to a direct effect of O_2 on the heart and to an increased parasympathetic tone, whereas the non- O_2 -dependent reduction in heart rate seems to be caused by a change in the beta-adrenergic stimulation of the heart.

B. Maximal work

5 Maximal oxygen uptake was significantly increased by 8-11 % at 1.4 ATA air, the increase being attributable to the rise in O_2 pressure. At ambient pressures in excess of 1.4 ATA, however, no further increase in $V_{O_2\max}$ was observed in spite of the additional rise in O_2 pressure. Instead $V_{O_2\max}$ at 2.0 and 3.0 ATA air was lower than at 1.4 ATA and at 6.0 ATA air was not significantly different from control at 1.0 ATA air. The observation that $V_{O_2\max}$ was significantly increased by 13 % at 3.0 ATA He- O_2 indicates that factors related to the increased N_2 pressure were responsible for the observed failure of $V_{O_2\max}$ to increase when the air pressure was raised beyond 1.4 ATA.

6 The maximal exercise ventilation decreased progressively as the ambient air pressure was raised. At 1.4 ATA the decrease averaged 7 %, at 2.0 ATA

18 % and at 6.0 ATA 46 %. The observation of a 14 % reduction in pulmonary ventilation at 3.0 ATA He-O₂ reflected the O₂-dependent part of the 33 % reduction in ventilation observed during air breathing at the same pressure.

7 The inadequate ventilatory response to maximal exercise at raised air pressures resulted in a progressive rise in end-tidal P_{CO₂} at the point of exhaustion. With He-O₂ at 3.0 ATA only a slight increase in end-tidal P_{CO₂} was observed corresponding to the O₂-dependent reduction in ventilation.

8 Maximal heart rate remained unchanged as the air pressure was raised to 1.4 ATA but then decreased at higher pressures. At 3.0 and 6.0 ATA air HR_{max} was significantly lower than control. During He-O breathing at 3.0 ATA HR_{max} was significantly lower than control but consistently higher than during air breathing at the same pressure.

9 Muscle metabolites (ATP, CP, lactate, pyruvate, glucose, glucose-6-phosphate), glycogen depletion and oxygen deficit at the point of exhaustion remained largely unchanged when the air pressure was raised to 1.4 ATA despite the significant increase in V_{O₂} max.

10 Peak blood lactate did not differ from control in any condition studied whereas endurance time was related to the ambient pressure in a manner similar to V_{O₂} max. This observation, together with the finding of a marked hyperoxia at 3.0 and 6.0 ATA air suggests that maximal work performance was more closely related to lactate concentration than to the P_{CO₂} or pH levels existing in the blood or in the working muscles at the point of exhaustion.

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**Dynamics of Pulmonary Gas Exchange
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at Start and End of Exercise**

by

Dag Linmarsson

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STOCKHOLM 1974

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I PREFACE

The present investigation is part of a research project dealing with the influences of various stress factors on the pulmonary gas exchange and circulatory functions. The experimental part of the investigation was carried out during the years 1972–1973

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Dag Linnarsson

Symbols and Abbreviations

Primary Symbols

V	Gas volume in general
\dot{V}	Gas volume per unit of time
P	Pressure in general including partial pressure
F	Fractional concentration in gas phase

Suffixes

I	Inspired gas
A	Alveolar gas
E	Expired gas
T	Tidal gas
ET	End-tidal gas
a	Arterial blood
\bar{v}	Mixed venous blood

Some other Symbols and Abbreviations

STPD	Standard temperature and pressure dry (0°C 760 mm Hg)
BTPS	Body temperature and pressure saturated with water vapor
BTPD	Body temperature and pressure dry
FRC	Functional residual capacity
HR	Heart rate
$\dot{V}O_2(FC)$	Rate of oxygen transfer between the alveolar space and the pulmonary capillaries volume per unit of time
V_L	Lung volume
WL	Work Load
AWL	Absolute work load
RWL	Relative work load
T_D	Time delay
a	Amplitude
τ	Time constant
MRT	Mean response time
l	Liter
mM	Millimoles per liter
min	Minute
sec	Second
msec	Millisecond
Hz	Cycles per second
W	Watt (1 W = 6.1 kpm/min)

Other symbols are defined in the text as they occur

II INTRODUCTION

The ultimate purpose of ventilation and circulation is to provide oxygen for the various energy consuming processes in the body and to remove the carbon dioxide formed during the same processes. During muscular exercise the O_2 need can be increased more than ten-fold compared to resting conditions. Despite such large changes in metabolic rate there is a remarkable constancy of the internal environment in terms of for example arterial blood gas concentrations during steady-state conditions. It is therefore generally agreed that the various respiratory and circulatory adjustments that accompany different intensities of physical activity are closely geared to the metabolic rate. Recent reviews concerning respiratory, circulatory and metabolic adjustments to muscular exercise include those of Asmussen (1965), Bevegård and Shepherd (1967), Edwards (1969) and Rowell (1974). Reviews on related topics may also be found in the proceedings of two recent symposia on "Physiology of Muscular Exercise" (edited by Chapman 1967) and "Muscle Metabolism during Exercise" (edited by Pernow and Saltin 1971).

Knowledge about the steady states of various physiological adjustments is generally insufficient in attempts to explain the regulatory mechanisms underlying the adaptational changes. Additional useful information may be found in the time patterns of the various changes involved and such information is increasingly used to study problems of fundamental importance in physiology. Much of our present knowledge about the physiology of exercise is derived from studies of the changes occurring during the unsteady states following onset of work or during recovery. Since the increased uptake of O_2 during exercise reflects the integrated action of a multitude of respiratory, circulatory and metabolic changes, measurements of changes in O_2 uptake, particularly in terms of O_2 deficit and O_2 debt, have been found to be useful tools in studies on exercise physiology. In 1913 Krogh and Lindhard used the changes in O_2 uptake during the initial stage of exercise to study the changes in pulmonary blood flow during exercise. The work of Margaria, Edwards and Dill (1933), introducing the concept of the alactacid and the lactacid portions of the O_2 debt, provides another example of an important contribution in exercise physiology based on measurements of O_2 uptake in the unsteady state. The analysis and presentation of experimental data in the study of Margaria *et al.* were greatly facilitated by the use of a mathematical model for the time course of O_2 uptake.

Traditionally interest in the time course of O_2 uptake has been focused primarily on the recovery period and the corresponding events at the onset of work have not been studied to the same extent. Berg (1947) published recordings of the response of O_2 uptake during onset of work and recovery but analyzed only the recovery responses.

by De Moor (1954) and Henry and De Moor (1956) showed that the increase of O_2 uptake in early exercise followed an exponential time course and Margaria *et al.* (1965) described this time course as a monoexponential function with a half time of about 30 sec. In view of the diversity of processes that are involved in the response to exercise such a monoexponential model appears surprisingly simple. It may be assumed that the results of earlier investigations were influenced by the lack of facilities for continuous measurements of O_2 uptake. It is therefore interesting that with the advent of new research tools such as fast response gas analyzers and computer facilities, breath-by-breath measurements of the pulmonary-capillary O_2 transfer made it possible for Auchincloss, Gilbert and Baule (1966) to confirm the observation by Krogh and Lindhard (1913) that O_2 uptake showed a sharp initial rise at the onset of exercise. Henry and De Moor (1956) and later Whipp and Wasserman (1972) using a breath-by-breath technique showed that the steady state O_2 uptake was appreciably delayed with heavy work. The notion that the slowing of the change in O_2 uptake depends on the relative rather than the absolute level of work finds support in the results of Linnarsson *et al.* (1974) who observed that when the aerobic work capacity was decreased with hypoxia the O_2 deficit for a given submaximal work load became increased. Correspondingly the increased aerobic work capacity during hyperoxia was found to be accompanied by a decreased O_2 deficit at the same submaximal work load. On the other hand Astrand and Saltin (1961) and Margaria *et al.* (1965) found that the maximal level of O_2 uptake was attained more rapidly the higher the external work load. With the use of various techniques and experimental conditions, the time course of O_2 uptake has thus been described as being either unchanged, slowed or accelerated with increasing work load. However, no systematic study seems to have been performed relating the directly measured time courses of O_2 uptake to the absolute and relative work intensity and to other cardiorespiratory functions that are known to be influenced by changes in work intensity.

In the study of the dynamic behavior of physiological functions the introduction of certain concepts used in control engineering has proven useful for example in studies on respiratory regulation (Grodins *et al.* 1954, Milhorn *et al.* 1965, Wigertz 1971, Reynolds and Milhorn 1973, Fujihara, Hildebrandt and Hildebrandt 1973a, b, Gelfand and Lambertsen 1973). A common approach has been to regard the physiological processes under study as a "black box" i.e. a system with unknown properties. Well-defined changes, e.g. in alveolar PCO_2 or work load are then used as input functions to the "black box". The time course of some dependent variable e.g. the pulmonary ventilation provides the output (response) of the system under study. By comparing the time patterns of the input and output functions, certain properties of the interposed system may be studied. On the basis of the relationship of output and input functions, mathematical models can often be used to describe the dynamic behavior of the system under study.

The limitation of the "black box" approach is that it can only describe what a system does, but not how it does it. The usefulness of this approach is primarily that hypotheses that are consistent with the characteristics of the system can be distinguished from hypotheses which are not (Fujihara *et al.* 1973a). Recalling the study of Margaria *et al.* (1933) their approach was in essence that of a systems analysis, although the terminology of control engineering was not used. Thus a step change in work load was used as the input function and the relationship between the time courses of the output and input functions was studied. The dynamic properties of the unknown system were described in terms of a mathematical model which was found to be consistent with the hypothesis that there are two compartments of the O_2 debt.

The purpose of the present investigation was to further analyze the time courses of respiratory gas exchange during the unsteady states of muscular exercise using recent advances in breath-by-breath recording technique and a systems analysis approach to quantify the time patterns during various conditions. More specifically the objectives of this work may be summarized as follows:

1. To further develop methods for the continuous breath-by-breath measurement of O_2 uptake and pulmonary capillary O_2 transfer during unsteady states of exercise and to study the time courses of these variables together with those of ventilation and heart rate by the use of analog and digital computation.
2. To identify and quantify the various time components of the pulmonary gas exchange and heart-rate responses during step changes in ergometric work load using a systems analysis approach.
3. To relate changes in the dynamic behavior of the measured variables to the work level in such a way that influences of the absolute work intensity could be distinguished from those of the relative work intensity.
4. To relate the various time components of the $\dot{V}O_2$ response to submaximal exercise to simultaneously measured ventilatory and heart rate responses.

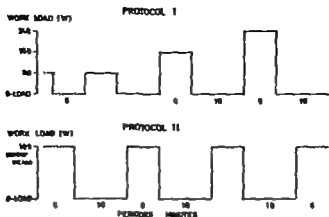


Fig. 1 Experimental protocols presented as work-load time profiles. Each of the protocols includes 3 positive and 3 negative step changes in work load. Each subject performed Protocol I on 3 occasions, breathing air and Protocol II with three different inspired gases: 14, 21 and 30 % O_2 in N_2 . The dotted lines denote warm-up periods.

separated by 10 min 0-load periods, the first 6 min of work being a warm-up period. For each subject a work load demanding approximately 65 per cent of $\dot{V}O_{2\max}$ during air breathing was selected. Each subject performed the Protocol II experiments once during air breathing (average $PiO_2 = 148$ mm Hg), once during inhalation of about 14 per cent O_2 in N_2 (average $PiO_2 = 99$ mm Hg) and once during inhalation of about 30 per cent O_2 in N_2 (average $PiO_2 = 214$ mm Hg). Thus from each of these experiments average responses to the same absolute work load could be obtained and then compared between normoxia, hypoxia and hyperoxia. Immediately following the last work period in the Protocol II experiments a period of maximal work was performed using a work load known to lead to exhaustion within 4–5 min during air breathing. $\dot{V}O_{2\max}$ was determined as above and post-exercise peak blood lactate concentrations were obtained from arterialized capillary samples from the fingertip and analyzed according to Barker and Summerson (1941) as modified by Ström (1949).

The subjects performed the experiments in a randomized order and they were not informed in advance about the exact nature of the exercise protocol or the composition of the inhaled gas. All experiments were started by 10 min of rest, with the subject breathing the preselected gas from the respiratory circuit. The following 6-min period of "warm-up" exercise completed the equilibration period. Throughout the experiments care was taken to ensure an environment free from disturbing noise or other extraneous influences.

Techniques Equipment

The general experimental arrangement is shown in Fig. 2. The subject was seated on an electrically braked *cycle ergometer* (Model AM 368 Elema, Sweden). The current through the load resistors was monitored and recorded to indicate the time and the nature of the work load (WL) changes. The work load could be changed without the subjects prior knowledge at exact preset time intervals using an electronic time relay. *Respiratory circuit*. In order to avoid dryness of the upper airways when breathing continuously in a mouthpiece during intervals of 1 hour or more and to achieve controlled humidity of respired gas, which is important for the accuracy of the $\dot{V}O_2$ computation heated and humidified gas was breathed from a 300 l neoprene bag (Camp Helsingborg Sweden). The inspired gas (air or N_2/O_2 mixtures containing about 14 and 30 per cent oxygen respectively) was supplied from pressure tanks at a rate sufficient to keep the inspiratory bag half filled during the experiments. In the bottom of the bag there was approximately 10 l of water. The water was heated by means of a brass tube through which water from a thermostating unit (Model FS Gebrüder Haake Berlin, FRG) was circulated. A blower circulated the inspired gas through wide bore externally insulated respiratory tubing (i.d. 38 mm) and from a T-connection a *respiratory valve* (dead space 35 ml) was connected. With this arrangement the inspired gas was saturated with water and, within narrow limits, kept at 37°C. Both the respiratory valve, the expiratory tubing and a *Venturi-type flowmeter* (Wigertz 1969) were heated by means of electrical heating tape (Electro-thermal Engn. Ltd.: London) in order to avoid condensation of water with ensuing volume changes of the expirate. At the outlet of the flow meter a low-resistance manifold was connected to allow the collection of expired gas in 300 l neoprene Douglas bags. The

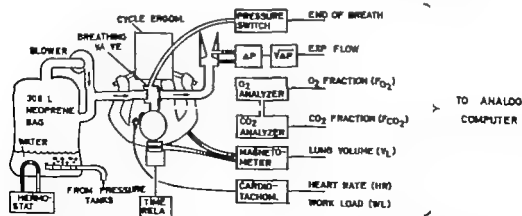


Fig. 2. General experimental arrangement. The inspiratory circuit is shown to the left of the subject sitting on a cycle ergometer whereas the various recording instruments are shown to the right. The symbols ΔP and $\sqrt{\Delta P}$ denote a differential pressure transducer and a square-root amplifier respectively.

pressure differential (ΔP) of the flow meter was measured using a differential pressure transducer (Model 270 with amplifier model 311 Sanborn Company Waltham Mass., USA) The ΔP of the Venturi flowmeter is proportional to the flow raised to the second power for which reason a square root amplifier was required to obtain a linear flow signal A pressure switch responding to the small pressure changes in the mouthpiece was used to detect the moment of termination of each breath. In the axial stream in the mouthpiece of the breathing valve a Teflon sampling tube was inserted (length 1.5 m I.d. 1.3 mm electrically heated to about 60°C) A sampling flow of 1 l/min was drawn by a vacuum pump (Model EDM 2, Edwards Ltd., Crawley England) through the sensing heads of a linear infrared CO_2 analyzer (Capnograph CG 119 Godart De Bilt Holland) and a fuel cell O_2 analyzer (Model 209 Westinghouse Corp., Pittsburg Pa USA) The logarithmic output of the O_2 analyzer was linearized using an antilogarithmic preamplifier (Sonis AB Sollentuna Sweden) In the sampling line between the analyzers and the vacuum pump a constant-flow regulator (Model DK 46 Krohne Duisburg FRG) was connected Lung volume (V_L) changes were monitored from changes in the anteroposterior diameters of the thorax and the abdomen using magnetometers as described by Mead *et al.* (1967) In this method two exciter coils generating a magnetic field are attached at the level of the nipples and the umbilicus on the anterior surface of the body and two receiver coils are attached to the surface of the back at corresponding levels An increase in the anteroposterior diameter results in a decrease of the intensity of the magnetic field and thus a decrease in the voltage induced in the receiver coils In order to avoid interference between chest and abdominal magnetic fields, different exciter frequencies are used By displacement of the pulmonary gas content from chest expansion to abdominal expansion while breath-holding (isovolume maneuver), the ratio between chest and abdominal movements for the same volume change can be determined for each subject Using this ratio to obtain equal weights with respect to volume changes, the two signals are added to obtain a signal with a linear relationship to changes in thoraco-abdominal volume (Mead *et al.* 1967 Gilbert *et al.* 1972) Heart rate (HR) was obtained from precordial ECG leads using a beat-to-beat heart rate meter with a linear analog output (Lindborg Wigertz and Ödman 1969)

Calibration of Transducers

The flowmeter was calibrated by having dry air pass the meter at a known constant rate (using a rotameter) with correction being made for the difference in density and hence in signal output between the dry air of room temperature and expired gas at 37°C (Wigertz 1969) The O_2 and CO_2 analyzers were calibrated with dry gases from pressurized cylinders the gases having been previously analyzed according to Scholander's micro

method. Since the expiratory circuit and the gas sampling line were heated to avoid condensation of water vapor and the analyzers have internal temperatures far exceeding the temperature of the expired gas, all volume and concentration measurements were made with gas having the same composition as during BTPS conditions, i.e. an amount of water vapor corresponding to the saturation pressure at body temperature was present to constitute about 6 per cent of the volume flow through the Venturi tube and correspondingly dilute the analyzed sample. Since the O_2 and CO_2 analyzers are not sensitive to water vapor *per se* (Linnarsson and Lindborg 1974) the FO_2 and FCO_2 readings obtained reflect the actual volume fractions of O_2 and CO_2 in the expirate and thus, multiplied by the barometric pressure (P_B) yield the corresponding partial pressures. It also follows that O_2 and CO_2 volumes that can be derived from the present volume and concentration measurements are to be expressed as volumes BTPD and multiplied by $P_B \times 273/760 (773 + 37) = P_B/863$ yield the corresponding STPD values.

The magnetometer output was calibrated against a known tidal volume by having the subjects breathe back and forth from a small plastic bag (volume 1.4 l) starting from functional residual capacity (FRC) and with a known volume of air in the bag. A previously determined FRC in sitting position for each subject provided a base-line value for this procedure yielding a calibrated V_L signal.

Analog On-Line Computations

An analog computer (PACE TR 48 Electronic Associates Inc. Princeton, N.J., USA) was connected on-line during the experiments. By means of standard circuits and operations (Carlsson *et al.* 1965) several computational procedures, such as integration, multiplication, peak value detection etc. could be performed simultaneously as is outlined on the flow diagram in Fig. 3. The continuous outputs from the various transducers were thereby transformed to one calibrated value per breath for each of the following variables: duration of each breath (T), pulmonary mid-volume (\bar{V}_L), end-expired values of FO_2 (F_{ETO_2}) and FCO_2 (F_{ETCO_2}), tidal volume (V_T) and the volumes of O_2 taken up (V_{TO_2}) and CO_2 eliminated (V_{TCO_2}).

The operation of a time integrator was started and terminated by the "end of breath" signal from the pressure switch, and thus a voltage was obtained proportional to breath duration. Similarly the various other computations were started and terminated for each breath.

The maximal and minimum values for lung volume in each breath as measured with the magnetometer were detected with peak following circuits and the arithmetic mean of these two volume readings were computed to obtain \bar{V}_L for each breath. End-expired

(peak) values of F_{O_2} and F_{CO_2} were also detected by means of peak following circuits in the analog computer

V_T , V_{TO_2} and V_{TCO_2} during each breath were computed as described by Linnarsson and Lindborg (1974). The theoretical basis for these computations is the following. The volume of O_2 taken up during one breath is the difference between the inspired volume of O_2 , $\int_0^T F_{IO_2} \text{ insp flow}(t) dt$ and the expired volume of O_2 , $\int_0^T F_{EO_2} \text{ exp flow}(t) dt$, where T is the duration of the breath. F_{IO_2} is the inspired and F_{EO_2} is the instantaneous expired fractional concentrations of O_2 . By making the assumption $\int_0^T \text{exp flow}(t) dt = \int_0^T \text{exp flow}(t) dt$ (eq 1) the more convenient relationship $V_{TO_2} = \int_0^T \Delta F_{O_2}(t) dt$ (eq 2) is obtained. Corresponding calculations for CO_2 yield V_{TCO_2} . The assumption in eq 1 that inspired and expired volumes are equal is incorrect when $V_{TO_2} \neq V_{TCO_2}$ and the respiratory exchange ratio (R) thus deviates from 1.0. This relationship introduces a potential error which requires subsequent correction in the computation of V_{TO_2} but not of V_{TCO_2} as long as the inspired F_{CO_2} is negligible.

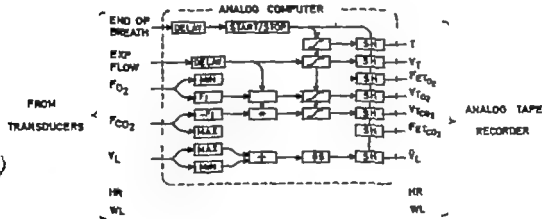


Fig. 3 Breath-by-breath on-line analog computations of (from above) duration of breath (T) total volume (V_T) end-tidal fractional concentration of O_2 (F_{ETO_2}), volume of O_2 taken up per breath (V_{TO_2}), volume of CO_2 eliminated per breath (V_{TCO_2}), end-tidal fractional concentration of CO_2 (F_{ETCO_2}) and pulmonary mid-volume (\bar{V}_L). The recordings of heart rate (HR) and work load (WL) are also indicated. The following symbols and abbreviations have been used to indicate the various steps in the computations. MIN = detect minimum value, MAX = detect maximum value F_1 = inspired fractional concentration $-$ = subtract, $+$ = add \times = multiply \int = integrate SH = sample hold. See Fig. 2 for explanation.

Signals from the rapid response transducers for flow \dot{V}_{O_2} and \dot{V}_{CO_2} were fed into the on-line connected analog computer. The flow signal was electronically delayed some 100 msec in order to attain time-alignment to the concentration signals which were subject to a corresponding delay due to the transport lag in the sampling tube. By comparing the instantaneous \dot{V}_{O_2} and \dot{V}_{CO_2} signals with preset F_{IO_2} and F_{ICO_2} voltage levels, voltages proportional to the differences between inspired and expired \dot{V}_{O_2} and \dot{V}_{CO_2} ($\Delta\dot{V}_{O_2}$ and $\Delta\dot{V}_{CO_2}$) were obtained. During expiration the expired flow and $\Delta\dot{V}_{O_2}$ signals were multiplied, and the resulting signal integrated over the duration of the breath to obtain a voltage proportional to V_{TO_2} in analogy with the computation of expired V_T through integration of expired flow.

The complete information for computing all the above variables was not available until a breath was terminated; thereafter the computations were performed during 5–10 msec and the resulting values were held and recorded during the ensuing breath. The output signals of the analog computer were recorded together with heart rate and work load on a multichannel FM tape recorder (Model FR 100 C Ampex Corp., Redwood City Calif., USA, tape speed 1 7/8 l.p.s.) and simultaneously displayed on a strip chart pen recorder (Mark 200 Brush Instruments Cleveland Ohio USA).

Calibration of the analog outputs for V_T (1 BTPS) and V_{TO_2} and V_{TCO_2} (1 BTPD) were obtained by adjustment of the gain factor of the integrators while supplying the flowmeter and the gas analyzers with gases of known flow and composition as described above (Calibration of Transducers). Impulses from a pulse generator with a frequency of 1/3 Hz were used to start and stop the computation of V_T , V_{TO_2} and V_{TCO_2} every 3 sec during calibration.

Digital Off Line Computations *

The stored data were subject to further off-line computations in a digital computer (IBM 1130). The play-back speed was four times the recording tape speed and A/D conversion (Interface 063 WDV Munich FRG) was made with a sampling frequency of 4/sec corresponding to 1 sec sampling intervals in experimental time.

For each sampling interval a number of computations were performed as is schematically outlined in Fig. 4. Barometric pressure (P_B) for each experiment was used to convert F_{ETO_2} and F_{ETCO_2} readings to partial pressures and to compute a factor ($P_B/863$) to convert O_2 and CO_2 volumes from BTPD to STPD. A value of V_T/n was subtracted from

* Detailed descriptions of the program software used in this study are available in the Library Karolinska Institutet, Solnavägen 1 S-104 01 Stockholm 60 Sweden.

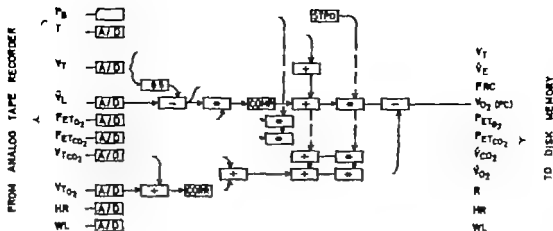


Fig. 4 Off-line digital computations using consecutive one-sec samples of the recorded variables (cf Fig. 3). The barometric pressure (P_B) for each experiment was also used for the computations. The following symbols and abbreviations have been used in addition to those listed in Fig. 3 to indicate the various steps in the computations: A/D = analog to digital conversion, + = divide, STPD = compute STPD conversion factor (p. 15), COMP = compare previous sample, CORR = compute correction factor for respiratory exchange ratio (p. 18). V_T , HR and WL undergo only A/D conversion. The new derived variables are (from above) ventilation (\dot{V}_E), functional residual capacity (FRC), pulmonary capillary O_2 transfer ($\dot{V}_{O_2}(PC)$), end-tidal partial pressures of O_2 (P_{ETCO_2}) and CO_2 (P_{ETCO_2}), carbon dioxide elimination (\dot{V}_{CO_2}), oxygen uptake (\dot{V}_{O_2}) and respiratory exchange ratio (R).

pulmonary mid-volume to obtain FRC . Respiratory exchange ratio (R) was computed as $R = R (1 - F_{IO_2}) / (1 - R F_{IO_2})$ where R is the ratio V_{TCO_2} / V_{TO_2} having the above mentioned error in V_{TO_2} when $R \neq 1.0$ (Linnarsson and Lindborg 1974). A corrected V_{TO_2} was thereafter computed as V_{TCO_2} / R . V_T , V_{TCO_2} and the corrected V_{TO_2} were divided by T to obtain \dot{V}_E , \dot{V}_{CO_2} and \dot{V}_{O_2} .

In order to compute the pulmonary-capillary O_2 transfer $\dot{V}_{O_2}(PC)$ (Auchincloss, Gilbert and Baule 1966) the breath-by-breath changes of the pulmonary O_2 stores were subtracted from \dot{V}_{O_2} as follows. The product $F_{ETCO_2} \times FRC$ for each sampling interval was computed and the corresponding value for the preceding sampling interval was subtracted. This difference was divided by T to obtain the change in pulmonary O_2 volume per unit of time which in turn was subtracted from the simultaneous \dot{V}_{O_2} value, the result being $\dot{V}_{O_2}(PC)$. Corresponding operations were performed to obtain pulmonary-capillary CO_2 transfer (not shown in diagram). The computed data were stored on a disk.

memory and, in the case of the Protocol I experiments together with results from the two other identical experiments for subsequent computation of individual mean responses

Computation of Individual Mean and Group Mean Responses

Respiratory and circulatory functions exhibit spontaneous fluctuations which will be superimposed as a "noise" on the basic response pattern during e.g. the adaptation to exercise and become noticeable when breath-by-breath or beat-by-beat recordings are made. In order to reduce this noise and correspondingly enhance the basic response pattern repeated identical experiments were averaged both for each individual and for the whole group of subjects. The background noise is reduced by a factor of \sqrt{n} where n is the number of determinations. In the case of group averaging also characteristic response patterns may be smoothed if they occur time scattered in relation to the stimulus. In this study the change in external work load

Individual mean time courses of $\dot{V}O_2$, $\dot{V}O_2(FC)$, $\dot{V}CO_2$, R , \dot{V}_E , \dot{V}_T , FRC , $P_{ET}O_2$, $P_{ET}CO_2$ and HR were computed using an IBM 1130 computer. For each subject condition and variable three on- and three off-responses were averaged, the averaging procedure being synchronized to the work load changes, i.e. each sample of the individual mean response is the average of three corresponding values of the recorded responses. For the Protocol I experiments averaging was done with data from identical transients of three different experiments while for the Protocol II experiments averaging was done with data from the three identical transients in one experiment.

The individual mean responses were stored on digital magnetic tape and the further data processing was performed using a large scale computer (IBM 360/75).

Individual values for the final levels of $\dot{V}O_2$, $\dot{V}O_2(FC)$, $\dot{V}CO_2$, R , \dot{V}_E , \dot{V}_T , FRC , $P_{ET}O_2$, $P_{ET}CO_2$ and HR were computed as time averages over the last 30 sec of work and over the last 2 min of O-load in each individual mean response. Mean values of these determinations are shown in Table III.

Group mean responses were computed from the eight individual mean-response data by the same principles as described above for the computation of individual mean responses. In this way a further reduction of random noise by a factor of $\sqrt{8}$ was obtained and at the same time the characteristic response patterns within the group were enhanced.

Estimation of Parameters for Quantitative Description of Time Courses

In order to facilitate the comparison between the time courses of a variable under different conditions and also to make comparisons between different variables, the time courses were described in terms of mathematical functions that were fitted to the experimental data. Since many of the variables under study (e.g. $\dot{V}O_2$, \dot{V}_E , HR) have been shown to behave like exponentially decaying functions of first or second order following step changes in external work load (Henry and De Moor 1956, Cerretelli, Sikand and Farhi 1966, Broman and Wigertz 1971, Whipp and Wasserman 1972) such functions were fitted to the recorded responses. In the mathematical models the amplitude of a response was expressed as a function of time. Thus the following functions of first order $f_1(t)$ and second order $f_2(t)$ were used

$$f_1(t) \begin{cases} 0 & \text{for } t < T_D \\ a_1 [1 - \exp(-(t - T_D)/\tau_1)] & \text{for } t > T_D \end{cases}$$

$$f_2(t) \begin{cases} 0 & \text{for } t < T_D \\ a_1 [1 - \exp(-(t - T_D)/\tau_1)] + a_2 [1 - \exp(-(t - T_D)/\tau_2)] & \text{for } t > T_D \end{cases}$$

where a_1 and a_2 are amplitude coefficients, τ_1 and τ_2 are time constants and T_D is the time delay which for the second-order function is the same for both terms of the function (Fig. 5). The amplitudes have the same dimension as the variable to be described by

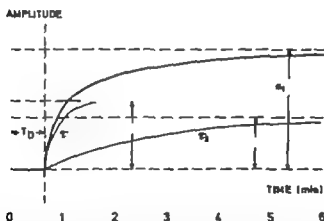


Fig. 5 Graphical representation of a second-order mathematical model employed to describe recorded responses, where the amplitude is expressed as a function of time (cf $f_2(t)$ p. 20). 0-time indicates the time for a step change in work load. The various function parameters used to characterize the response are indicated in the figure, where T_D = time delay before the onset of the response, τ_1 and τ_2 = time constants for each of the two terms of the function, a_1 and a_2 = the corresponding amplitudes when $t \rightarrow \infty$. In this example T_D is 40 sec and τ_1 and τ_2 are 15 and 120 sec respectively.

the function whereas τ_1 , τ_2 and T_D are expressed in units of time. One time constant is equal to the time when $(1 - e^{-1}) \times 100 \approx 63$ per cent of the final change has taken place. For comparison the half time for a function is equal to $0.69 \times \tau$ and the term rate constant used by Whipp (1971) among others denotes the inverse of τ . Ultimately when $t = \infty$ the change is equal to a_1 for the first-order function and to $a_1 + a_2$ for the second order function. For practical purposes it may be noted that after the slowest time constant has elapsed 5 times, only 0.7 per cent of the final change remains until the steady state value is reached. In some of the second-order functions where the steady-state value was not reached during the 6–10 min of recording, $a_1 + a_2$ exceeded the difference between the final observed values of the on and off-responses listed in Table III.

The computation of best-fit function parameters was made using a least-squares method. *Le* the function parameters were chosen so as to minimize the sum of the squared differences between the fitted function and the recorded response (loss function, L). To minimize L , an iterative algorithm was utilized in the present study. The method was similar to that used by Broman and Wigertz (1971) and was further modified accord

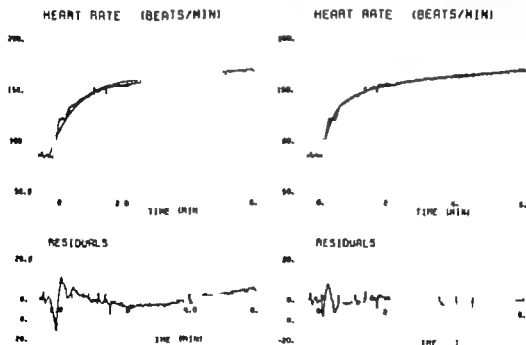


Fig. 6 Individual mean on-response of heart rate to a step change in work load between 0-load and 240 W together with first-order (left) and second-order (right) best-fit functions. The superior fitting of the second-order function is clearly shown. Note notch in the heart-rate response approximately 10–20 sec after start of work. Residuals (lower tracing) denote the vertical distance between the fitted function and the recorded responses as a function of time

ing to Marquardt (1963) This fitting procedure can be carried out without knowledge of the final steady state value of a response and it operates efficiently even in the presence of noise and spontaneous fluctuations superimposed on the basic response signal For each variable experimental condition and subject and for the corresponding group averages mono- and biexponential functions were fitted to the recorded responses, starting 30 sec before a change in work load and continuing until the next shift in work load

An example of best fit mono- and biexponential functions is shown graphically in Fig 6 together with recorded data and the differences between recorded response and the fitted function as a function of time Certain criteria were used to judge whether an experimental response was better described by a second-order than by a first-order function a) The loss function had to be appreciably lower when the second-order model was applied b) In case a negative time delay resulted with the first-order model this delay had to be less negative with a second-order model c) The two time constants of the second-order model should be distinctly different d) Group averaging should reduce L approximately in proportion to the number of subjects by reducing random noise provided the applied model is a good description of the measured response If L is only slightly reduced during group averaging this implies that the order of the applied model is too low

Plotting of individual and group mean responses and best fit functions was done with a CALCOMP 835 plotter coupled to an IBM 360/75 computer and using standard sub-routines

Computation of Mean Response Time O_2 Deficit and O_2 Debt

A simple measure of the rate of adjustment of a variable is the mean response time (MRT) For a first-order function this is the sum of the time delay (T_D) and the time constant (τ_1) The MRT value can be used for comparisons of the overall rate of change between responses to which first-order functions can be fitted

By definition the deficit for a response for example the O_2 deficit is equal to the area between the final or steady state level and the measured response throughout the work period Since in the fitting procedure the fitted curve follows the measured response so that the sum of areas between the two curves becomes zero the area between the final level and the fitted function is also equal to the deficit The corresponding condition holds true for the off responses Deficit and debt values can thus be obtained as the product of MRT and the amplitude of a response (Whipp 1971) when true steady-state values are reached during the recording period For those work loads where a true steady state was not attained the level of O_2 need used to compute the deficit was

defined as the mean value over the last 30 sec of work at each particular work load in agreement with the definition of O_2 deficit used by Hansen (1934). The O_2 need during exercise in hypoxia was defined as being equal to the final value of $\dot{V}O_2$ obtained with the same work load during air breathing. In those recovery responses where the base-line $\dot{V}O_2$ was not reached within 10 min debt was computed from the area between the best-fit function and base-line $\dot{V}O_2$ until they converged.

Statistics

Conventional statistics were applied. Intralindividual differences were evaluated using Student's *t*-test (Fischer 1946).

however the variable exhibited a continuous change throughout the measuring period although slower towards the end. Therefore since all the values of Table III are not true steady state values, the term final value has been used.

Oxygen uptake during work increased linearly with the absolute work load in the graded AWL experiments. On the basis of \dot{V}_{O_2} values of the last 30 sec the relative work load (RWL, Table II) averaged 31, 54 and 78 per cent respectively at 80, 160 and 240 W. The final values for \dot{V}_{O_2} during air breathing in the Protocol II experiments (mean 2.57 l/min STPD) agreed closely with what could be predicted from the same average work load and using the \dot{V}_{O_2} /work-load relationship of the Protocol I experiments. During the inhalation of 14 % O_2 in N_2 the final \dot{V}_{O_2} value was significantly reduced by 8 per cent ($p < 0.01$) while no significant difference in \dot{V}_{O_2} was observed between air and 30 % O_2 in N_2 . Using the final value for \dot{V}_{O_2} in the air experiments to determine the aerobic cost of work for each subject during all the three experimental conditions of the constant AWL study the mean relative work load (Table II) amounted to 64 per cent breathing air and to 80 and 60 per cent respectively with 14 % O_2 and 30 % O_2 . During the last 2 min of 0-load \dot{V}_{O_2} averaged 0.56 l/min STPD and did not vary with the preceding work load except after very heavy work, where 0-load \dot{V}_{O_2} amounted to 0.61 l/min STPD.

Carbon dioxide elimination during 0-load averaged 0.47 l/min STPD in the graded-AWL study whereas 0.03–0.07 l/min STPD lower values were observed in the constant AWL experiments.

Respiratory exchange ratio during exercise increased with increasing relative work load to average about 0.97 with very heavy work and with hypoxia. During 0-load in the graded AWL experiments the lowest R values were found following the highest work load. Zero-load R was consistently lower during the constant AWL than during the graded AWL experiments.

Ventilation during exercise varied roughly in proportion to the relative work load from a mean value of 31 l/min BTPS at an RWL of 31 per cent to 93 l/min BTPS at an RWL of 80 per cent. Corresponding changes were observed for tidal volume except for hypoxia where V_T was lower than during very heavy work despite largely the same average V_E .

Functional residual capacity during work decreased with increasing RWL. During 0-load FRC averaged 3.30 l BTPS and did not differ between the various conditions.

End-expired P_{O_2} in all the air experiments averaged 102 mm Hg during exercise and was 2–6 mm Hg higher during 0-load. Similarly with 30 % O_2 the P_{ETO_2} was higher during 0-load (172 mm Hg) than during work (165 mm Hg). With 14 % O_2 however 4 mm Hg lower values were obtained during 0-load where P_{ETO_2} averaged 62 mm Hg.

Mean end-expired P_{CO_2} during work ranged from 40.1 to 42.7 mm Hg in the air

breathing experiments and was slightly higher with 30 % O_2 . With hypoxia P_{ETCO_2} was significantly lower by 9 mm Hg ($p < 0.001$) compared to air with constant AWL. Similarly P_{ETCO_2} during O-load was significantly reduced with hypoxia ($p < 0.001$)

Exercise heart rate increased in an approximately linear fashion with absolute and relative work load in the graded AWL experiments. In the constant AWL experiments the highest mean value was found with 14 % O_2 while exercise HR did not differ significantly between air and 30 % O_2 and exceeded by 6 and 12 beats/min respectively the HR that would be predicted from the HR/RWL relationship of the graded AWL experiments. HR during the last 3 min of O-load was increased after exercise at 240 W compared with corresponding values after 80 and 160 W. In the constant AWL-experiments O-load HR following a given RWL was 3–5 beats/min higher than in the graded AWL experiments.

Configuration of Responses

The group mean responses of $\dot{V}O_2$, $\dot{V}CO_2$, R , \dot{V}_E , \dot{V}_T , FRC, P_{ETO_2} , P_{ETCO_2} and HR are shown in Fig 7–13. Individual time courses have been recorded for each variable step response and condition. Essentially all the typical response patterns of the individual recordings could be found also in the group response recordings (see further "Intra and Interindividual Averaging" p. 46). Close inspection of the curves reveals that the changes in HR started immediately whereas in the recordings of respiratory variables the first changes could be observed only after a few seconds delay which coincides with the inherent delays of the recording procedure.

For $\dot{V}O_2$ three distinctly different phases could be recognized both in the individual and in the group mean on-responses. Typically during the first 10–15 sec period there was a rapid increase followed by levelling-off during 5–10 sec at a level which for example in the 240 W experiments amounted to 1.2 l/min STPD. Thereafter a rapid secondary change of large amplitude was observed being complete within 1.5–2 min. At work loads of 199 W and above a third phase resembling a slow upward drift could be discerned. The corresponding three phases could be identified in the individual and group mean off-responses of $\dot{V}O_2$. With the exception of a higher amplitude of the first phase the response pattern of $\dot{V}O_2(PC)$ agreed well with that of $\dot{V}O_2$ (Fig. 16).

\dot{V}_E showed an initial rapid response of a relatively modest amplitude followed by a secondary change accounting for the major part of response. This pattern could also be identified in the \dot{V}_T recordings and in the on responses most of the initial change of \dot{V}_T was accounted for by a rapid initial decrease in the FRC level.

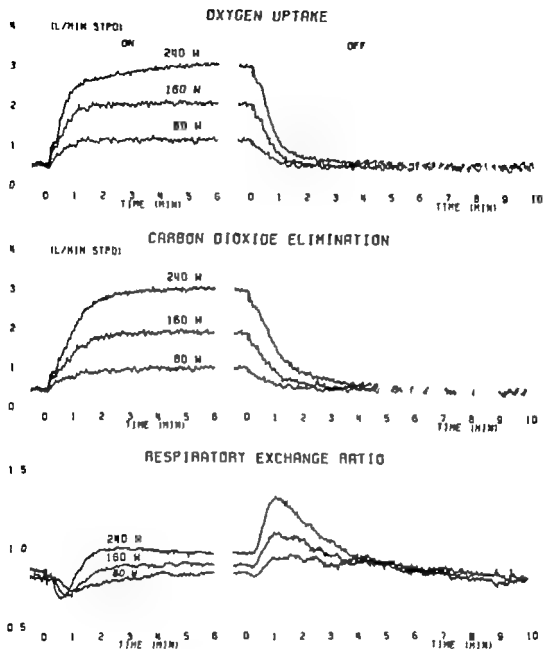


Fig. 7 Graphical representation of group mean responses of O_2 uptake, CO_2 elimination and respiratory exchange ratio to light (80 W), moderate (160 W) and very heavy (240 W) work, breathing air. Responses from both the transition between loadless pedalling and exercise (on) and the reverse transition (off) are shown. Each single tracing computed from breath-by-breath recordings from 24 experiments in 8 subjects. 0-time denotes the time for the change in work load.

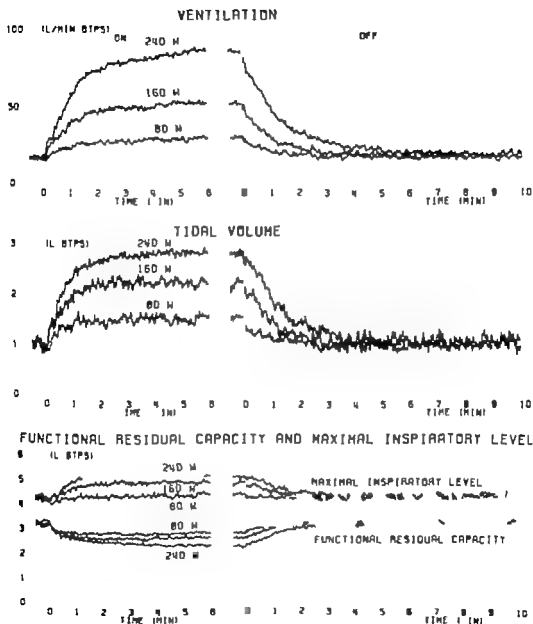


Fig. 8. Group mean on- and off-responses of ventilation, tidal volume and functional residual capacity to graded submaximal work, breathing air. Maximal inspiratory level is also shown, and was obtained by summing V_T and FRC responses. Same experimental conditions as in Fig. 7.

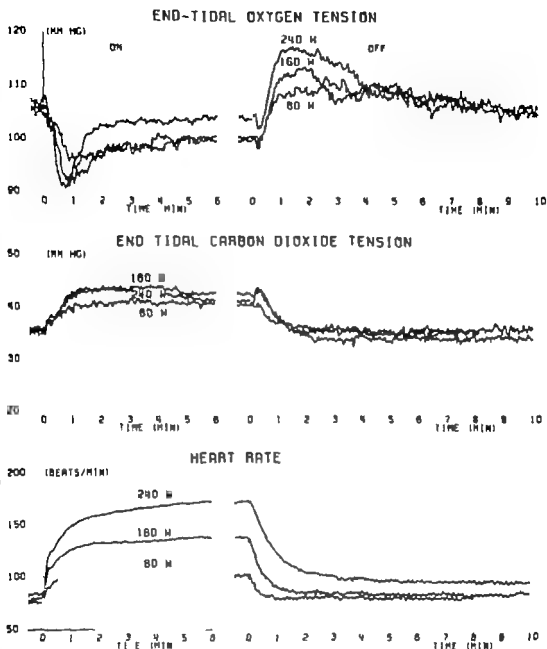


Fig. 9 Group mean on- and off-responses of end-tidal PO_2 and PCO_2 and heart rate to graded submaximal work breathing air. Same experimental conditions as in Fig. 7

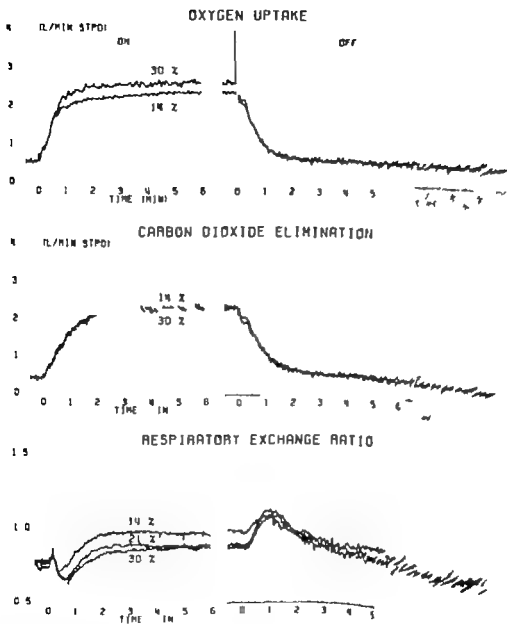


Fig. 10 Group mean on- and off-responses of O_2 uptake, CO_2 clearance ratio to exercise of work load demanding in each subject about 65 per cent breathing. Tracings refer to experiments with 14, 21 and 30 per cent O_2 . Data from the 21 % O_2 experiments have been omitted in the single tracing computed from breath-by-breath recordings from 24 subjects. The vertical line at 0 minutes denotes the time for step change in work load.

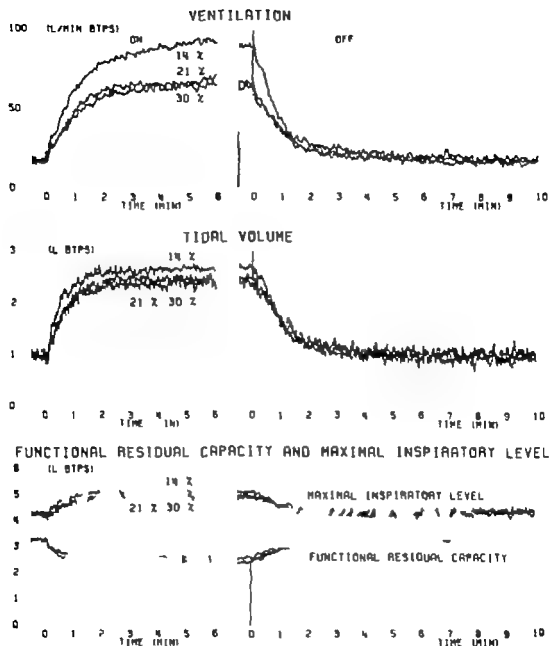
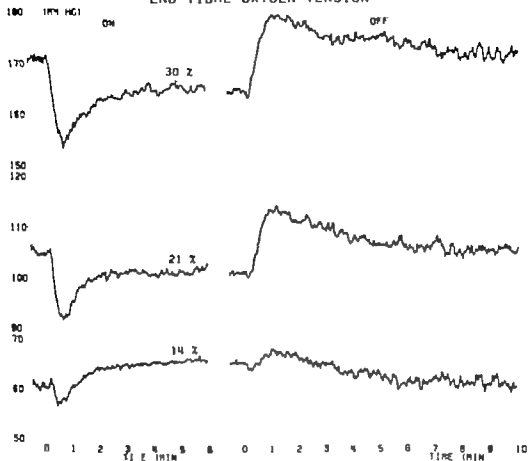


Fig. 11 Group mean on- and off-responses of ventilation, tidal volume, functional residual capacity and maximal inspiratory level to constant submaximal exercise with hypoxia, normoxia and hyperoxia. Same experimental conditions as in Fig. 10

END TIDAL OXYGEN TENSION



END TIDAL CARBON DIOXIDE TENSION

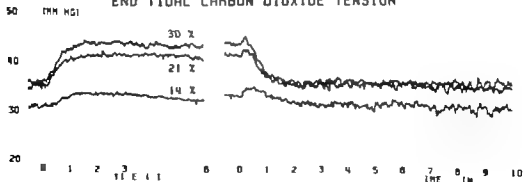


Fig. 12 Group mean on- and off-responses of end-tidal PO_2 and PCO_2 to constant submaximal work with hypoxia, normoxia and hyperoxia. Same experimental conditions as in Fig. 10

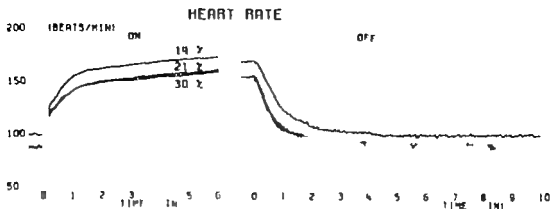


Fig. 13 Group mean responses of heart rate to constant submaximal work with hypoxia, normoxia and hyperoxia. Same experimental conditions as in Fig. 10

Parameterization of Responses

In order to express the configuration of the various recorded responses in quantitative terms and thereby allow comparisons and statistical analyses among the various time courses, model functions were fitted to the recorded responses. The object of this quantitation procedure has been primarily to describe the overall rates of change and to identify whether slow secondary changes appear in addition to the major initial responses (see further "Model Functions" p. 46).

The fitting procedure and the criteria used to determine whether a first- or a second-order model comprised the best description of a recorded time course have been described above (p. 22). Function parameters (amplitudes a_1 , a_2 , time constants, τ_1 , τ_2 , time delays T_D) of best fit functions for the various group mean responses are given in Table IV-VI. For a first-order function the three parameters a_1 , τ_1 and T_D are given and for a second-order function two additional parameters, a_2 and τ_2 are included (cf. Fig. 5).

First-Order Functions were found to describe the time courses of respiratory variables adequately during the on- and off-responses to exercise at the lower range of RWL. Accordingly, with light and moderate work and breathing 30% O_2 (RWL < 60 per cent) \dot{V}_{O_2} and $V_{O_2(FC)}$ adjusted monoexponentially both in the on- and off-transients. The V_E on responses to light and moderate work and with constant AWL breathing air or 30% O_2 could be fitted with first-order models while this was the case for the off-responses only with light work and with 30% O_2 . In these and other responses described as being of first order the use of a second-order model did not result in a better fitting between the measured responses and the applied functions.

Table IV Parameterization (cf Fig. 5) of best-fit first or second-order functions for group mean responses of \dot{V}_{O_2} uptake pulmonary-capillary \dot{V}_{O_2} transfer \dot{V}_{CO_2} elimination and respiratory exchange ratio. Data refer to transients following onset of work (on) and recovery (off) at absolute work loads of 80 160 and 240 W breathing air (graded AWL) and at a constant AWL (group mean 199 W) performed at inspired O_2 concentrations of 14 21 and 30 % respectively (τ_1 τ_2 and T_D values given in sec).

		on					off				
		τ_1	1	τ_2	2	T_D	τ_1	1	τ_2	2	T_D
<u>\dot{V}_{O_2} uptake, \dot{V}_{max} STPD</u>											
21 % O_2	80 W	0.67	29.4			9.3	-0.44	37.1			1.5
	160 W	1.34	32.5			8.1	1.37	37.8			3.4
	240 W	2.12	38.5	2.71	1980	7.8	-3.30	33.4	-0.23	710	9.9
14 % O_2		1.62	29.3	0.77	930	6.0	1.74	33.5	1.25	4140	12.2
21 % O_2	Constant	1.78	26.7	2.42	3830	10.9	1.99	38.1			5.2
30 % O_2	AWL	2.01	33.4			9.3	2.06	48.7			3.9
<u>Pulmonary capillary \dot{V}_{O_2} transfer, \dot{V}_{max} STPD</u>											
21 % O_2	80 W	0.89	28.1			1.5	-0.70	34.0			-7.3
	160 W	1.63	33.3			2.5	1.60	38.1			0.3
	240 W	2.81	31.2	0.69	220	-1	-2.16	27.0	-0.24	352	10.9
14 % O_2		1.44	23.8	0.46	148	3.8	1.70	29.0	2.61	8720	12.4
21 % O_2	Constant	1.85	31.2	2.35	4160	4.1	1.93	34.6			7
30 % O_2	AWL	2.13	34.2			1.9	2.11	38.4			3.0
<u>\dot{V}_{CO_2} elimination, \dot{V}_{max} STPD</u>											
21 % O_2	80 W	0.53	53.4			5.4	-0.54	50.9			3.1
	160 W	1.48	52.8			7.4	1.35	48.5	-0.24	482	0.8
	240 W	2.39	54.4			9.1	2.39	48.2	-0.82	2590	2.9
1 % O_2		1.96	54.2			.8	88	42.4	1.18	3800	12.2
21 % O_2	Constant	1.87	53.0			7.9	1.80	53.3	-0.78	3880	2.9
30 % O_2	AWL	1.88	57.2			6.2	1.87	57.7			1.9
<u>Respiratory exchange ratio</u>											
21 % O_2	80 W	-0.62	43.1	0.60	3.9	9.5	0.3	20.1	1.63	5270	27.0
	160 W	1.34	20.7	1.32	29	24.4	0.31	19.8	-0.40	179	23.2
	240 W	2.88	16.3	3.03	20.3	21	0.48	21.7	-0.87	129	23.7
14 % O_2		0.23	20.1			49.2	0.34	17.7	-0.7	22	28.3
21 % O_2	Constant	42	17.3	.53	24.3	20.9	0.1	8	-0.53	1.8	23.0
30 % O_2	AWL	-8.47	14.3	0.53	40.3	21.2	0.29	4.7	-0.36	161	22

First-Order Time Constants ranging between approximately 20 sec and 70 sec were found. For both on- and off-responses of \dot{V}_{O_2} and $\dot{V}_{O_2}(PC)$, τ was about 30 sec. \dot{V}_{CO_2} changed at a considerably slower rate, the τ_1 values being about 50–60 sec. Corresponding values for \dot{V}_E ranged between 60 and 70 sec.

Table V Parameterization of best-fit functions for group mean responses of ventilation, tidal volume and functional residual capacity for symbols and experimental conditions, see Table IV

		on					off				
		τ_1	1	τ_2	2	T_D	1	1	τ_2	2	T_D
Ventilation l/min STPS											
21 % O ₂	80 W	12.7	60.1			5.6	12.4	51.7			-6.0
	160 W	35.0	61.3			3.5	28.8	48.3	7.87	308	1.9
	240 W	63.3	62.3	42.3	2450	8.8	56.5	65.9	12.9	304	2.0
14 % O ₂	Constant AWL	61.7	58.8	22.6	425	2.2	-64.6	45.1	-41.3	3160	5.0
21 % O ₂		51.2	311			2.6	-45.7	39.1	-6.6	346	-0.6
30 % O ₂		48.8	68.6			1.0	-45.6	66.7			1.7
Tidal volume, l STPS											
21 % O ₂	80 W	0.51	31.3			9.5	-0.18	1.2	-0.22	41.0	1.3
	160 W	1.22	40.6			8.1	1.20	61.0			2.2
	240 W	1.82	44.5			4.4	1.77	78.7			9.0
14 % O ₂	Constant AWL	0.86	11.0	1.01	43.5	4.7	1.66	52.4			12.8
21 % O ₂		1.49	44.2			1.9	1.48	70.5			5.7
30 % O ₂		1.39	34.8			5.8	1.40	70.9			8.0
Functional residual capacity l STPS											
21 % O ₂	80 W	-0.30	14.5			3.1	0.36	43.3			5.0
	160 W	-0.57	24.1			3.1	0.62	63.			0.4
	240 W	-0.52	10.4	0.48	132	2.5	0.96	92.7			1.0
14 % O ₂	Constant AWL	-0.34	18.7	-0.3	77	4.5	0.67	72.6			4.3
21 % O ₂		-0.34	5.1	-0.57	53.9	3.1	0.58	51.9	0.32	210	1.9
30 % O ₂		-0.16	2.8	-0.55	38.1	4.3	0.69	74.9			1.3

Both *First and Second-Order Functions* could be fitted to the on- and off-responses of HR, to the $\dot{V}O_2$, $\dot{V}O_{2(PC)}$ and \dot{V}_L responses in the higher range of RWL and to the on-responses of FRC. For those responses the use of a second-order model resulted in a better fit between the measured time course and the applied function. This is graphically shown in Fig. 6 where an individual on-response of HR is shown together with best fit functions of first and second order. For biphasic changes such as the on- and off-responses of R and P_{ETCO_2} the superior fit of second-order models was particularly evident and the L values frequently differed from those of the first-order fit by a factor of 10 or more.

Second-Order Time Constants generally consisted of one rapid time constant dominating the initial part of the response and a slower component dominating the later part of the response. τ values considerably exceeding the duration of the recorded on (360 sec) and the off (480–600 sec) responses are associated with a greater error in their determination compared with response components reaching their steady-state values within

Table VI Parameterization of best-fit functions for group mean responses of end-tidal PO_2 and PCO_2 and heart rate. For symbols and experimental conditions, see Table IV

		m					mT				
		1	1	τ_2	2	T_D	τ_1	1	τ_2	2	T_D
<u>End-tidal O_2 tension, mm Hg</u>											
21 % O_2	80 W	-29.6	41.9	22.3	81.9	7	9.9	22.5	-35.5	6490	20.5
	160 W	-53.2	21.8	37.8	39.4	19.2	16.	22.5	12.4	300	22.1
	340 W	-76	15.8	75.5	25.1	18.6	21.4	26.6	20.8	197	23.8
14 % O_2	Constant	-7.2	6.5	12.3	32.6	20.0	7.5	31.0	11.7	126	31.8
21 % O_2		-79.5	16.7	74.7	24.1	19.0	18.3	22.1	1.6	148	22.9
20 % O_2		32.8	16.3	27.9	46.9	19.0	16.6	18.6	-11.5	278	22.2
<u>End-tidal CO_2 tension, mm Hg</u>											
21 % O_2	80 W	3.0	34.5			1.8	2.5	8.3	3.0	104	21.4
	160 W	7.5	23.7			17.3	-7.4	30.1			25.2
	340 W	9.2	31.2	25.0	2150	13.0	-7.9	34.9			34.0
14 % O_2	Constant	4.4	43.5	11.1	1020	25.1	7.5	31.0	-11.7	126	31.8
21 % O_2		6.4	25.1			16.2	-6.1	22.4	12.7	7100	31.7
20 % O_2		7.8	23.8			17.9	-8.3	28.3			28.1
<u>Heart rate, beats/min</u>											
21 % O_2	80 W	32.9	11.2	3.9	367	1.7	22.3	15.9			2.1
	160 W	49.7	22.4	44.6	1510	1.8	-51.6	25.5	-4.1	199	2.3
	340 W	57.6	20.3	34.7	143	1.0	-69.4	45.9	9.7	378	6.7
14 % O_2	Constant	57.1	26.8	49.2	851	-0.8	-56.8	44.2	12.8	107	7.3
21 % O_2		81.8	23.2	38.2	354	1.7	-45.7	27.0	30.1	113	5.1
20 % O_2		52.9	20.3	29.6	377	1.7	-54.7	31.7	10.0	253	2.9

the recording period. Thus the slow components of e.g. the VO_2 on responses to constant AWL and very heavy work breathing air ($\tau_2 \approx 2000$ sec or more) can just as well be described as being linear drifts, having a more rapid rate for the higher work load. For HR the rapid component had a time constant of about 70 sec, followed by a slow component accounting for a larger portion of the response the higher the work load.

Time Delays. Most responses to work-load changes took place after an initial delay ranging from a few sec to half a min. A short delay corresponding to the duration of one breath (approx. 2–3 sec) is inherent in the computation of the respiratory variables. For the heart rate recordings, the corresponding recording delay amounted to 0.5–1 sec.

The fitting procedure may result in T_D estimates not found by close inspection of the recorded responses, since T_D and the other function parameters from a mathematical point of view merely represent adjustment parameters to obtain best fit during the whole response period. Especially in cases where the order of the selected exponential function is too low, differences between estimated and recorded time delays may be found. A small negative T_D may thus result when a rapid initial change has a large amplitude. This

frequently occurred in the HR on-responses, and became in all cases much less pronounced when a second-order model was applied (cf Fig 6). In the same way the occurrence of an immediate change of only a small magnitude as in many respiratory variables and in the off responses of HR at high work loads may result in a short time delay in the fitted function.

The time delays of the order 15–30 sec found in almost all $R_{P_{ET}O_2}$ and $P_{ET}CO_2$ responses are of such a magnitude that they cannot be ascribed to the recording or fitting procedures. Thus in these variables no or only slight changes took place during the first 15–30 sec following a change in work load.

Mean Response Time (MRT for definition see p 22) represents the time until the "center of gravity" for a change has taken place and is synonymous with the term mean transit time commonly used in connection with dye dilution studies of blood flow. Since the parameter MRT is an estimate of the overall rate of change for a fitted response it can be used to compare the responses between various variables and conditions (cf Table VII). In order to show the degree of dispersion of individual rates of response and to demonstrate the agreement between the means of individual and group mean responses, MRT values from group mean responses have been listed together with the means of individual determinations and their standard errors. A good agreement was found between means of individual values and the MRT values of the group mean responses with some few exceptions at the lowest work load. The same good agreement was found for the other function parameters when comparing means of individual parameters and the corresponding group mean values. This may be taken to indicate that any of these two ways to characterize the "average" individual can be used. The most rapid overall changes were found in the on-responses of HR during light work (MRT = 15.1 sec) and the slowest changes were found in FRC during recovery after very heavy work (MRT = 91.7 sec).

Comparisons Between Overall On- and Off-Responses

The general tendency was that variables and conditions exhibiting a slow on-response also showed a slow off-response. However with the exception of light work, certain differences between the dynamic behavior of on- and off-responses were noted for some of the variables, and the levels of significance of intraindividual differences between on- and off-values for MRT are listed in Table VIII. With 14 % O_2 MRT for \dot{V}_{O_2} was prolonged by 7 sec in the off response. For \dot{V}_{O_2} no other differences were found between overall on-

Table VII. Mean response times (sec) for on- and off-transients of cardiorespiratory variables. Data refer to submaximal exercise at baseline work loads (AWL) of 80, 160 and 240 W breathing air and at constant AWL (group mean 199 W) performed at inspired O_2 concentrations of 14, 21 and 30 %, respectively. Values from group mean responses (GMR) are given together with mean values (M) and standard errors of mean (SEM) of individual determinations in eight subjects for $\dot{V}O_2$, $\dot{V}O_2(FC)$, $\dot{V}E$, $\dot{V}T$, FRC and HR. For symbols, see Table III

			on						off					
			$\dot{V}O_2$	$\dot{V}O_2(FC)$	$\dot{V}E$	$\dot{V}T$	FRC	HR	$\dot{V}O_2$	$\dot{V}O_2(FC)$	$\dot{V}E$	$\dot{V}T$	FRC	HR
80 W	↑	21 % O_2	GM	38.7	39.4	59.0	65.7	40.8	21.6	15.1				
			M	39.4	33.4	67.2	81.1	39.3	21.5	15.6				
			SEM	2.4	4.1	11.9	19.1	6.8	3.6	1.6				
160 W	↑	21 % O_2	GM	42.6	30.8	60.2	64.8	43.7	27.2	28.4				
			M	42.6	29.7	66.4	65.1	43.7	21.3	28.9				
			SEM	1.6	1.9	3.3	4.3	4.6	3.5	2.7				
240 W	↑	21 % O_2	GM	45.4	41.9	62.6	74.2	48.9	42.3	39.7				
			M	43.9	41.8	62.3	74.3	49.9	44.9	40.3				
			SEM	1.4	1	2.1	2.1	6.9	8.6	2.9				
14 % O_2	↓	21 % O_2	GM	1.8	40.0	29.0	71.4	32.7	29.1	48.0				
			M	47.4	39.1	38.3	71.6	32.1	28.9	48.3				
			SEM	1.3	1.9	2.4	8.3	3.4	5.3	3.8				
21 % O_2	↓	21 % O_2	GM	42.0	40.1	60.9	63.9	46.1	29.3	38.2				
			M	42.3	39.7	60.6	60.2	43.0	29.9	38.1				
			SEM	1.7	2.4	2.4	5.0	3.1	7.0	4.1				
30 % O_2	↓	21 % O_2	GM	42.6	36.1	43.8	70.6	40.3	32.8	32.1				
			M	43.8	35.1	44.0	70.6	41.2	32.8	32.9				
			SEM	1.7	1.4	3.3	3.3	3.7	7.4	3.9				

Table VIII Levels of significance for differences between on- and off responses of $\dot{V}O_2$, $\dot{V}CO_2$, V_E , V_T , FRC, and HR. Data refer to submaximal exercise at absolute work loads (AWL) of 80, 160 and 240 W breathing air and at a constant AWL (group mean 199 W) performed at inspired O_2 concentrations of 14, 21, 30 %, respectively. 0 = not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ respectively. For symbols, see Table III.

		$\dot{V}O_2$	$\dot{V}CO_2$	V_E	V_T	FRC	HR
21 % O_2	80 W	0	0	0	0	0	0
	160 W	0	0	0			0
	240 W	0		0			
14 % O_2	Constant		0	0			
21 % O_2	AWL	0	0	0			
30 % O_2	AWL	0	0	0			

Table IX. Levels of significance for differences between various experimental conditions, with regard to the mean response times for on- and off-transients of $\dot{V}O_2$, $\dot{V}CO_2$, V_E , V_T , FRC and HR. Data refer to submaximal exercise at absolute work loads (AWL) of 80, 160 and 240 W breathing air and at a constant AWL (group mean 199 W) performed at inspired O_2 concentrations of 14, 21 and 30 % respectively. For symbols, see Table III, VIII

on							off						
		$\dot{V}O_2$	$\dot{V}CO_2$	V_E	V_T	FRC	HR	$\dot{V}O_2$	$\dot{V}CO_2$	V_E	V_T	FRC	HR
21% O_2	80 W	0	0	0		0		0				0	***
	160 W		0		0	0				***			
	240 W		0	0	0							0	
14% O_2	Constant	0		0		0	0		0	0	0	0	
21% O_2	AWL	0	0	0	0	0	0	0	0	0	0	0	0
30% O_2	AWL	0	0	0	0	0	0	0	0		0	0	

and off-responses. In conditions where RWL averaged > 60 per cent the recovery of HR was prolonged compared to the on-response. In contrast to V_E which did not differ in this respect between on and off, V_T and FRC changed more slowly during recovery than following onset.

Changes in Overall Responses with Different Work Loads and Inspired O_2 Tensions

In the experiments with graded AWL, V_{O_2} did not differ between light and moderate work with respect to the overall rates of change in the on- and off responses (Table IX). By contrast, MRT with very heavy work was significantly prolonged both in the on- and off responses of V_{O_2} due to the contribution of a second slower component to the response pattern. V_E , V_T and FRC responses seemed to be slowed under the influence of an increasing work load to a larger extent during the off responses than during the on responses. When the RWL was varied by using a constant AWL and varying the inspired P_{IO_2} , no consistent changes in the overall rates of on responses were observed despite changes in RWL of about the same magnitude as the difference between the two highest loads in the graded AWL experiments. During recovery however, the rates of change of both HR and V_{O_2} were significantly slowed with hypoxia compared to normoxia and hyperoxia.

V DISCUSSION

A Methodological Considerations

Maximal O_2 Uptake

Duplicate determinations of $\dot{V}O_{2\max}$ using the present technique have in this laboratory been shown to agree within 2 per cent at both normal and increased P_{IO_2} . The changes in $\dot{V}O_{2\max}$ obtained with hypoxia (-20 per cent) and hyperoxia (+7 per cent) in the present study agree well with previous findings (Fagnaeus *et al.* 1973 Linnarsson *et al.* 1974) from experiments where P_{IO_2} was altered by changing the ambient air pressure. In the present study particular care was taken to ascertain a constant and known F_{IO_2} both during maximal and submaximal work. The recording of respired FO_2 allowed F_{IO_2} to be continuously monitored throughout the experiments. In addition F_{IO_2} was verified in samples drawn from the inspiratory part of the breathing valve and subsequently analyzed with the Scholander technique.

Breath-by-Breath Measurements during Submaximal Work

$\dot{V}O_2$, $\dot{V}CO_2$ and R. The accuracy of the breath-by-breath measurements of $\dot{V}O_2$, $\dot{V}CO_2$ and R, using basically the present technique, has been studied previously (Linnarsson and Lindborg 1974) and breath-by-breath determinations of $\dot{V}O_2$ and $\dot{V}CO_2$ were found to agree with simultaneous Douglas technique measurements within 0.02 l/min STPD during submaximal exercise. In the present study some of the computational procedures previously performed with analog technique (division by breath duration, computation of R) have been performed digitally which may have increased the accuracy of the computations slightly.

Lung Volume. Determinations of V_L using the magnetometer technique have been reported to agree well with simultaneous spirometric determinations (Mead *et al.* 1967), Grimby, Bunn and Mead 1968). FRC values determined during quiet breathing in the sitting position were used as a calibration base line. Using body surface measurements to monitor respiratory mid-volume and to compute FRC by subtracting $V_T/2$, the absolute level of FRC is only properly determined if the nongaseous content of the thorax including blood remains constant. There is clear evidence of a redistribution of blood volume from the legs towards the thorax at the transition from rest to exercise in the upright position (for review see Gauer and Thron 1965) but a corresponding decrease in residual volume of the lungs has not been observed (Assmusen and Christensen 1939). On the contrary Hanson, Tabakin and Caldwell (1962) found a slight increase in total lung capacity

and residual volume in the transition from motionless standing to level walk. The same authors also found an unchanged residual volume comparing level walk with walking at 4° inclination. It is therefore concluded that the procedure used in the present study to assess lung volume changes adequately reflects the actual changes in FRC

End Tidal PO_2 and PCO_2 End-tidal PO_2 and PCO_2 are widely used as convenient indices of arterial blood gas tensions during exercise. For both O_2 and CO_2 however end tidal estimates may differ from arterial determinations. Such end-tidal to arterial differences may arise if a gas sample obtained at the end of expiration is not representative for the average composition of alveolar gas during a breath. This mechanism was proposed by Matell (1963) to explain the findings of lower end-tidal than arterial PCO_2 values during rest and the reverse difference during exercise in the following way. During rest the alveolar sample is contaminated with dead-space gas while during exercise the increased metabolic rate and the forced breathing give rise to larger cyclic fluctuations of alveolar PCO_2 with the highest values at the end of the breath. For O_2 the same mechanism would lead to lower end tidal than mean alveolar values during exercise. Mean alveolar O_2 tension may in turn appreciably exceed arterial values due to ventilation/perfusion differences. The interaction of these influences on the arterial to end tidal differences of PO_2 and PCO_2 has been analyzed by Rosenhamer (1972) and under conditions similar to the present experiments P_aCO_2 during light exercise exceeded P_{ETCO_2} by approximately 2 mm Hg while P_{ETCO_2} was 1 mm Hg higher than P_aCO_2 during moderate exercise. It is therefore likely that the large differences in P_{ETCO_2} between O-load and exercise in the present study to a large extent do not reflect corresponding differences in arterial PCO_2 . For PO_2 end-tidal to arterial differences of 8–15 mm Hg were found for light and moderate exercise with P_{ETO_2} values closely approximating the PO_2 of an ideal alveolar gas sample.

Computation of Pulmonary-Capillary O_2 and CO_2 Transfer A rapid increase in respiratory mid-level would increase the pulmonary O_2 store through two mechanisms, both because the volume of the alveolar space is increased and because an increased amount of inspired gas with a higher PO_2 is added. It was thought of interest to determine to what degree changes in pulmonary O_2 stores were included in the O_2 uptake as measured at the mouth, and therefore the pulmonary-capillary O_2 transfer was computed breath by breath. Since the depth of inspiration is subject to irregular fluctuations in normal breathing (Hlastala, Wranne and Lenfant 1973) a high level of "noise" will be introduced into a breath-by-breath recording of the O_2 stores and into a variable such as $V_{O_2}(PC)$ where changes in O_2 stores are included. Although the typical response pattern for $V_{O_2}(PC)$ in the early portion of a response could be discerned despite the "noisy" character of recordings, it was not possible to establish whether or not secondary slow components of low am

plitude were present in individual recordings. The $\dot{V}O_{2(FC)}$ recordings therefore did not lend themselves to analysis of intraindividual differences in overall rates of response.

From a theoretical standpoint breath by-breath changes in pulmonary CO_2 stores may be of minor importance for the time course of \dot{V}_{CO_2} for two reasons. First the volume of CO_2 in the alveolar space is very small compared to other rapidly changing CO_2 stores of the body (cf Farhi and Rahn 1955). Second changes in CO_2 volume and concentration due to a change in respiratory mid level tend to cancel each other. Accordingly no differences were found between the recording of \dot{V}_{CO_2} and pulmonary-capillary CO_2 transfer in a pilot study and this variable was not further studied.

Experimental Conditions

Inspired O_2 Tensions. The inspired O_2 tensions in the present hypoxic and hyperoxic experiments were selected to create conditions similar to those in experiments by Linnarsson *et al.* (1974) who studied muscle metabolites during submaximal and maximal work and found that the O_2 deficit and lactate accumulation varied inversely with PiO_2 in the range of 100–200 mm Hg.

Work Loads. Zero-load pedalling was used as a convenient base-line condition. By having the subject pedal at the same rate during the entire experiment the work load could be changed without the subject's prior knowledge thereby avoiding anticipatory influences on the respiratory and circulatory response patterns. It was also thought that with the use of 0-load instead of rest between the work periods, the time needed for recovery could be shortened allowing a larger number of successive transients to be studied within the given period of time. About one hour was found to be the longest experimental duration that could be used without causing any appreciable discomfort to the subjects.

The term zero load is a misnomer in the sense that a metabolic load is in fact imposed on the subject under 0-load conditions. In the present study $\dot{V}O_2$ during zero load averaged 0.56 l/min STPD. Extrapolation from the $AWL/\dot{V}O_2$ relationship in the 80 and 160 W experiments suggests that about 20 W is performed during 0-load which is considerably less than the value 45 W obtained by Beaver and Wasserman (1968) but agrees well with what can be calculated from data presented by Whipp and Wasserman (1969). The underlying assumptions for the above extrapolations remain however uncertain. For the purpose of the present study 0-load is merely considered to be the mildest form of ergometric exercise possible with a pedalling rate of 60 rpm.

The work intensities in the graded AWL experiments (80, 160 and 240 W) were chosen to cover a wide range of submaximal work loads during air breathing. A work load for each project corresponding to about 65 per cent of $\dot{V}O_{2max}$ during air breathing was

selected for the constant AWL experiments. This work load was considered high enough to result in a significant lactate accumulation in the working muscles during air breathing (Karlsson 1971), and low enough to avoid a maximal work situation when the same external work load is performed in hypoxia where $\dot{V}O_{2\max}$ is known to be decreased (cf Faguraeus *et al.* 1973)

For all subjects the relative work loads during air breathing were computed as the ratio between actual $\dot{V}O_2$ during the last 30 sec of work and $\dot{V}O_{2\max}$. The $\dot{V}O_2$ values for submaximal work with air were considered to indicate the O_2 need for each particular work load and subject and accordingly RWL for the experiments with hypoxia was computed as the ratio between $\dot{V}O_2$ during air breathing and $\dot{V}O_{2\max}$ during hypoxia. Using the lower submaximal $\dot{V}O_2$ values obtained during the hypoxic experiments would have given too low RWL values due to the slowed response of $\dot{V}O_2$ during hypoxia.

Work Patterns. In recent years various work profiles such as sinusoidal step ramp function and impulse work-load changes (Stegemann 1958 Beaver and Wasserman 1968 Wiggertz 1971 Fujikura Hildebrandt and Hildebrandt 1973a, b) have been used to study the dynamics of cardiorespiratory adaptation to exercise. In the present study a step-change work-load pattern was used due to its simplicity both in regard to its application, the subsequent analysis and the way the results can be directly visualized. A six min work interval agrees well with what is commonly used in exercise testing and was chosen as a compromise between various considerations. Limitations in the data storage capacity on one hand, set a limit for the total time available for the experiments and on the other hand a certain number of repeated experiments was necessary to obtain the desired degree of noise reduction. A third consideration was that the duration of each response must be long enough to allow proper determination of the dominating time components.

Similar considerations were made also for the recovery period and it was also attempted to minimize the influence of one work period on the following by having the highest work load at the end of Protocol I (graded AWL). Adequate recovery of $\dot{V}O_2$ was found 8 and 10 min after the 80 and 160 W experiments respectively and 10 min after work in the Protocol II experiments (constant AWL). A comparison between the HR before the onset of the 80 W work load and the 240 W work load (mean HR 76 and 84 beats/min respectively) on the other hand indicates a slight change with time in the base-line condition. For a given work period the relative intensity of the preceding work period had the highest values in the constant AWL experiments, notably those with hypoxia, where also the largest changes were found for base-line HR, mean values being 96 and 104 respectively during the last 30 sec of the first and third 0-load periods.

The R value at the end of a recovery period can also be used to indicate whether basal conditions of respiratory gas exchange have been achieved. During severe exercise leading

to accumulation of fixed acids. CO_2 stores in the body are mobilized and blown off from the lungs due to excessive hyperpnea. Following the initial rise in \dot{V}_E during recovery, sub-normal \dot{V}_E values can be obtained when CO_2 stores are refilled. This relationship has been illustrated by Hansen (1934) and may explain why recovery \dot{V}_E values were slightly lower following repeated heavy exercise in the constant AWL experiments than following a comparable work load in the graded AWL experiments where only one very heavy work period was performed in each experiment.

Parameterization and Data Reduction

Model Functions. Various mathematical models for the description of cardiorespiratory responses have been proposed and these have recently been reviewed by Fujihara *et al.* (1973b). Since the fitting of mathematical models to the recorded responses in this study was used to describe the overall responses rather than to detect all the involved time components, the simple model of Wigertz (1970) was used, consisting of a time delay followed by one or two exponential changes starting at the same time. This model has been proven useful also to detect the secondary slow changes in the responses (Broman and Wigertz 1971) which was one of the main objectives in this study.

Intra- and Interindividual Averaging. The theory underlying the averaging procedure has been presented above (p. 19). Averaging of repeated individual respiratory responses to repeated identical stimuli has been used *i.e.* by Beaver and Wasserman (1968), Fujihara *et al.* (1973a, b) and Reynolds and Milhorn (1973) in order to make the basic response pattern stand out more clearly. It was found in these studies, that the basic features of each response were enhanced by the averaging procedure, indicating that the response pattern was related in a constant way to the stimulus (work-load change). This was seen also in the present study when comparing the recorded and the averaged individual responses. One typical example is the characteristic "notch" in the HR on-response (Fig. 6) which remained apparent in the recordings before and after averaging. The same HR pattern can also be used to illustrate the consequences of intraindividual averaging by comparing Fig. 6 and Fig. 9. Evidently the temporal dispersion of this HR pattern within the group of subjects was small enough to make the individual pattern appear with only slight modification in the group mean responses. Similar conclusions both in regard to intra- and interindividual responses can be made from other typical response features *e.g.* the rapid initial drop in the FRC on response or the delay in the responses of \dot{V}_E and $\text{P}_{\text{ET}}\text{O}_2$. Therefore in the present study it has been considered justified to analyze both individual and group mean responses and since essentially all information from the individuals can be found in the group mean data, mainly the latter have been selected to illustrate the results.

U General Discussion

Dynamic Behavior of \dot{V}_E , V_T and FRC in Response to Step Changes in Work Load

The ventilatory responses to exercise resembled those described by among others Asmussen and Nielsen (1948), Dejours (1959) and Matell (1963) showing an initial rapid response occurring without delay and followed by a secondary slower change starting some 20–30 sec later. This response pattern was found uniformly in the subjects and was often found more distinctly in the off-responses of V_E (Fig. 8.11). The responses to moderate and very heavy work differed from those reported by Dejours (1959) and Matell (1963) in that the magnitude of the initial component was smaller and less abrupt. According to Dejours (1959) about half of the ventilatory response can be ascribed to the initial component but in the present study the average magnitude of the initial component exceeded 25 % of the final ventilatory level only during light work.

The possibility that important transients may not be evident in total ventilation but appear in V_T or respiratory rate has been pointed out by Dejours (1967). Accordingly V_T on- and off transients were studied. In addition recordings of FRC were used to analyze whether changes in V_T were accomplished through changes in the inspiratory or expiratory reserve volumes (Fig. 8.11). In the on-responses V_T exhibited more distinctly the same basic response pattern as \dot{V}_E , i.e. a rapid initial component followed after about 20 sec by a secondary slower change. There was a slight transient drop in V_T immediately before the appearance of the slower component. The initial component could be accounted for almost entirely by a rapid drop in the FRC level. This may be taken to indicate that the immediate respiratory response to the onset of dynamic leg exercise is an increase in expiratory activity. At the same time the maximal inspiratory level remained almost unchanged (Fig. 8). According to Grimby, Dunn and Mead (1968) a decrease in FRC is associated with a corresponding decrease in the abdominal diameter. Since the abdominal muscles are abruptly engaged at the onset of dynamic leg exercise a simple mechanical relationship between leg and trunk movements may be of some importance for the initial changes in FRC level. The secondary slower V_T increase mainly consisted of an increase in the maximal inspiratory level. The final values of maximal inspiratory level and FRC (Fig. 8.11) agree well with previous findings by Asmussen and Christensen (1939) that the increase in V_T is taken to a similar degree from the inspiratory and the expiratory reserve volumes.

The overall response of V_T was markedly slower during recovery than following onset of work (Table VIII, Fig. 8.11) and was associated with an equally slow time course of FRC towards pre-exercise values. The difference in time course between the on- and off changes in FRC suggests that the factors underlying the increased expiratory activity

effected through a withdrawal of vagal tone for example during isometric muscle contraction (Freyschuss 1970). In a recent study of the initial HR response to dynamic exercise (Fagraeus and Linnarsson 1974) it was found that also in this situation vagal withdrawal is the primary factor responsible for the rapid phase of the cardioacceleration. Secondary to this rapid phase there is a further slow and continuous increase in heart rate (Broman and Wigertz 1971) with a concomitant slow increase in cardiac output (Rochester *et al.* 1961, Grimby Nilsson and Sanne 1966, Cerretelli *et al.* 1966, Gilbert Auchincloss and Baule 1967) which is more marked at higher work loads (Jones *et al.* 1970).

The HR responses observed in the present study (Fig. 9-13) agreed largely with those described by previous investigators. Close inspection of individual and group mean curves reveals however that a more precise description of the on responses should include three components: (1) a very rapid change during the first 10-15 sec, (2) a slower rise during the following 60-90 sec and (3) a slow almost linear drift (Table VI) lasting throughout the work period. In most individual HR recordings a levelling off or more often a temporary decrease in HR was observed following the initial rapid rise but before the secondary slower increase (Fig. 6-9-13). Such a negative component in the HR response can be discerned in the recordings of Craig, Cummings and Blevins (1963) and has also been described by Beaver and Wasserman (1968) and Fujihara *et al.* (1973a, b), who concluded that the "notch" in the on-response of HR is synchronized with the change in work load and is not merely a continuation of the sinus arrhythmia of the base-line condition. The notch in the HR response is abolished by atropine but not by β -adrenergic blockade (Fagraeus and Linnarsson 1974) which suggests that the initial vagal withdrawal during the first 10 sec is followed by a transient increase in vagal tone of about the same duration. The physiological mechanism underlying these biphasic changes in the vagal influence on the heart is not known.

The magnitude of the initial component of the HR on response increased with the work-load in the graded AWL experiments (Fig. 9). The share of the two slower components, however, increased proportionally more from 30 per cent of the final 4 min response with light work to about 50 per cent with very heavy work. This observation which is in agreement with the findings of Broman and Wigertz (1971) implies that the overall HR response is retarded with increasing work load (Table VII-IX). With moderate and very heavy exercise the slowest HR component could be described as a linear drift amounting to 5 and 14 beats/min respectively during the last 4 min of exercise. For comparison it may be noted that during clinical exercise testing HR is generally considered to have achieved a relative steady state if it does not change more than 10 beats/min from the second to the sixth min of exercise (Sjostrand 1967).

In the constant AWL experiments the on-responses of HR showed almost identical patterns with hypoxia, normoxia and hyperoxia (Fig. 13, Table IX). In the case of

hypoxia however the response curve was shifted on the average 12 beats/min upwards (Table III) It thus seems that for the on-response of HR the rate of adjustment is determined by the absolute rather than the relative work load Hypoxia seems to cause a shift in the "set point level" for HR and this shift appears to be independent of AWL since it did not differ between 0-load and heavy work An increase in $P_{ET}O_2$ of about 65 mm Hg induced by substituting 30 % O_2 in N_2 for air affected neither the rate of change nor the absolute levels of HR during the present experiments

The observation that the overall on response of HR became slower with increasing sub maximal AWL may at first sight seem to be at variance with the findings of Åstrand and Saltin (1961), that HR increases more rapidly the heavier the work load imposed during a maximal work test The findings of Åstrand and Saltin may however indicate that the amplitude of the rapid component of the HR response continues to rise with AWL also during supramaximal work, accounting for almost the whole change from base-line to maximal HR during extremely heavy work thereby leaving no room for the slower components

The off-response of HR did not exhibit any fluctuations of the type observed in the on-response but resembled closely a second-order exponential function (Fig. 9 13) The overall off responses were slower than the corresponding on-responses (Table VIII) which is in agreement with the observations of Gilbert *et al.* (1967) and Broman and Wigeritz (1971) An interesting observation was that in the off-responses in hypoxia and very heavy work, the high HR persisted for some sec before the rapid drop occurred (Table VI) This phenomenon has been observed also in maximal exercise (Di Prampero Peeters and Margaria 1973).

The overall rates of change in the off-responses of HR were significantly slowed both with increasing AWL in the graded AWL experiments and with increasing RWL in the constant AWL experiments (Table IX) which may be taken to indicate that the overall rate of the HR change during recovery is determined by the relative and not by the absolute work load the latter being the case in the ventilatory responses and the on-responses of HR There is evidence that the metabolic situation in the muscles is involved in the regulation of the HR response to muscular exercise possibly through the action of peripheral receptors (Stegemann 1963 Arnesen *et al.* 1965 Stegemann and Kenner 1971) The present results are compatible with such a hypothesis, since the metabolic changes induced by varying the inspired P_{O_2} and thereby the RWL in constant-load experiments are likely to be much more pronounced at the end than immediately following the start of the exercise period This would explain why the rate of HR change at the start depends on AWL, while the corresponding rate of adjustment during recovery seems to depend on RWL.

Interrelation Between Ventilatory and Heart Rate Dynamics

A dissociated dynamic behavior of ventilatory and circulatory responses to muscular exercise has previously been observed (Wade and Bishop 1962 Wigertz 1970 Broman and Wigertz 1971). Such a dissociation would be expected to cause disturbances in the balance between the delivery of O_2 to the lungs and the removal of O_2 from the lungs by circulation with ensuing changes in alveolar gas composition. Also in the present study marked differences between the overall rates of change were noted between V_E and HR (Table VII). During the first minutes following a change in work load large transient changes were noted for $P_{ET}O_2$ (Fig. 9-12) indicating a transient relative hypoventilation in early exercise and a transient relative hyperventilation during recovery. However during the initial 15–30 sec following the start or end of work the end tidal gas tensions remained

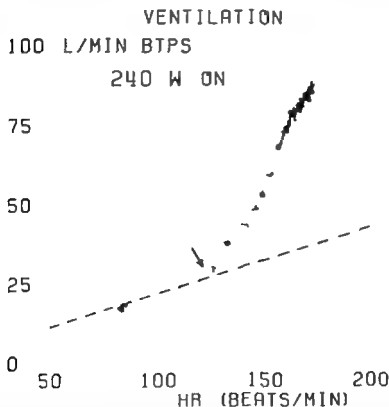


Fig. 14 Relationship between group mean responses of ventilation and heart rate following start of exercise at a work load of 240 W. Small dots represent consecutive one-sec samples with each 30-sec sample indicated by a circle. The cluster of dots at bottom left refers to the last 30 sec of the base-line condition prior to the onset of work. Arrow indicates when the first 15 sec of work have elapsed. Broken line indicates equal changes of the two variables in per cent of their base-line values.

relatively unchanged. A remarkable constancy during the first 30 sec of exercise has been observed also in continuous measurements of arterial blood gases and pH (Matell 1963, Barr *et al* 1964, Bjurstedt and Wigertz 1971). The ventilatory and circulatory changes occurring during this interval thus seem to be fairly well synchronized which also became evident when the changes relative to the base-line condition for \dot{V}_E and HR were compared. Thus both \dot{V}_E and HR 15 sec after the onset of work at a load of 240 W comprised about 150 per cent of the base-line value. This relationship is illustrated in Fig. 14 where group mean responses of \dot{V}_E and HR have been plotted second by second in an x-y diagram. It is therefore tempting to postulate a common regulatory mechanism for \dot{V}_E and HR during the initial phase of exercise. Since a change in the FRC level seems to account for the rapid initial phase of ventilatory response (Fig. 8) group mean FRC and HR changes were compared in an x-y diagram (Fig. 15). The rectilinear relationship in the diagram indicates almost identical time courses which suggests a common regulatory mechanism.

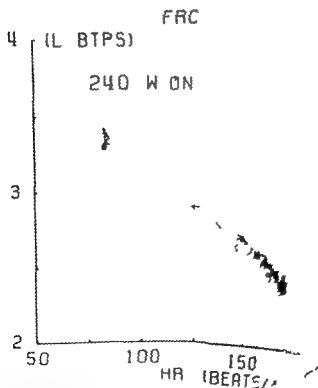


Fig. 15 Relationship between group mean responses of functional residual capacity (FRC) and heart rate (HR) at the onset of exercise. The work load of 240 W is indicated. The values are shown at top left.

Dynamic Behavior of $\dot{V}O_2$ in Response to Step Changes in Work Load

A survey of some earlier studies on the time courses of $\dot{V}O_2$ following the onset and end of muscular exercise has been given above (see Introduction p 7). The present study is mainly concerned with two aspects of the dynamics of $\dot{V}O_2$: first to describe as accurately as possible the various components of the time course of $\dot{V}O_2$ in the unsteady state and second to relate the time course of $\dot{V}O_2$ to the absolute and relative work levels and to the time courses of some other physiological variables.

Of the three phases which can be distinguished in the time course of $\dot{V}O_2$ in the unsteady state of exercise (p 27 Fig. 7-10) the first phase seems to be related to the rapid change in blood flow through the lungs that takes place immediately upon a change in work load. This relationship between circulation and O_2 uptake was pointed out by Krogh and Lindhard (1912-1913) but it was not until half a century later that the initial rapid increase of the pulmonary-capillary O_2 transfer at the onset of work was actually recorded by Auchincloss, Gilbert and Baule (1966). These authors, who used an advanced computer-oriented technique to measure $\dot{V}O_2$ breath by breath and to compensate for changes in pulmonary O_2 stores, also showed that variations in the breathing pattern following onset of work do not greatly alter the time course of $\dot{V}O_{2(PC)}$, the pulmonary O_2 stores providing a buffer between the O_2 intake to the lungs and the pulmonary-capillary O_2 transfer. Since the subjects in the present study exhibited a good synchronization between the initial responses of \dot{V}_E and HR (Fig. 14), $\dot{V}O_2$ and $\dot{V}O_{2(PC)}$ displayed similar time courses in early exercise. The magnitude of the initial rapid component of $\dot{V}O_{2(PC)}$ amounted to 0.85 l O_2 per min at the onset of work at a load of 240 W (Fig. 16) com-

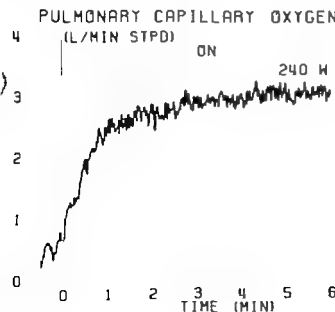


Fig. III Group mean response of pulmonary-capillary O_2 transfer at the onset of exercise at a work load of 240 W during air breathing. Tracing computed from breath-by-breath recordings from 24 experiments in 8 subjects.

PULMONARY CAPILLARY OXYGEN TRANSFER

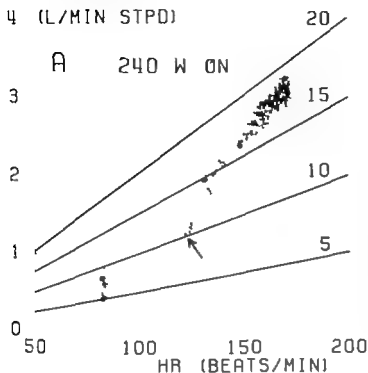
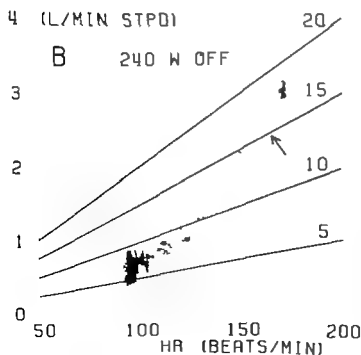


Fig. 17 A Relationship between group mean responses of pulmonary-capillary O_2 transfer and heart rate following the onset of exercise. O_2 -pulse isopleths (ml O_2 per heart beat) are indicated. Dots, circles and arrow as in Fig. 14



B Relationship between group mean O_2 -responses of same variables as in A. Note that start values appear at top right.

prising about 30 per cent of the final 6 min response. The interrelationship between the time courses of $\dot{V}O_2(PC)$ and HR is depicted in an x-y diagram in Fig. 17 (cf Davies 1968) where also O_2 -pulse isopleths have been included. During the first 15-sec period changes in stroke volume and/or in the arterial to mixed venous O_2 difference resulted in a relatively modest increase in the O_2 pulse from about 7 to 10 ml O_2 per heart beat. Since some increase in stroke volume can be assumed to take place during the first 15 sec following the start of work in the upright position (Saltin *et al.* 1971) only slight changes in the $(a-\bar{v}) O_2$ difference are likely to have taken place during this period. The temporary relative constancy of the $(a-\bar{v}) O_2$ difference may be characteristic only for the transition between 0-load and exercise or for the transition between different work loads, since Edwards *et al.* (1972) observed that mixed venous $P\dot{V}O_2$ ($P\dot{V}O_2$) exhibited an early pronounced fall being almost complete within the first 15 sec following the transition from motionless rest to dynamic leg work of moderate intensity. These authors ascribed the fall in $P\dot{V}O_2$ to a sudden surge of blood with a low O_2 saturation from stagnant pools in the legs due to the activation of the muscle pump. No such pools are likely to have occurred in the present study where 0-load pedalling was used as the base-line condition which may account for the apparent discrepancy between the present results and those of Edwards *et al.* (1972). The body position may also affect the time courses of the various adjustments following start and end of work. Thus in the supine position the stroke volume has been reported to show a transient decrease immediately following the start of work (Raynaud *et al.* 1973) and a transient increase during recovery (Cumming 1972). It can therefore be concluded that the greatest magnitude of the first phase of the $\dot{V}O_2(PC)$ response should be expected in the transition from motionless rest to exercise in the upright position, whereas the corresponding response should be expected to be of considerably smaller magnitude in the transition between 0-load and work in the supine position.

The rapid change in the O_2 pulse observed 15 sec after the onset of work (Fig. 17) corresponds to the *second phase* in the $\dot{V}O_2(PC)$ and $\dot{V}O_2$ responses. Since most of the stroke volume change can be assumed to have taken place during the initial 15 sec (cf Saltin *et al.* 1971) a rapid widening of the $(a-\bar{v}) O_2$ difference is likely to be the cause of the further increase in O_2 pulse. The delay until the widened $(a-\bar{v}) O_2$ difference caused an increase in $\dot{V}O_2(PC)$ presumably resulted from the time required for the transport of blood from the exercising muscles to the lungs. The second phase of the $\dot{V}O_2(PC)$ and $\dot{V}O_2$ responses was found to be completed within 1.5–2 min following the start of work presumably corresponding to the time necessary for the redistribution of blood flow to and within the working muscles. Support for this assumption can be found in the results of Clausen and Lassen (1971) who found by the use of intramuscular depots of radioactive xenon that the increase in blood flow in the exercising muscles was completed within the first min of exercise.

During light and moderate work steady-state values of $\dot{V}O_2$ and $\dot{V}O_{2(PC)}$ were attained when the second phase was completed *i.e.* about 1.5–2 min after the start or the end of work. Fitting a first-order model to the data obtained during light and moderate work, a mean response time for $\dot{V}O_2$ of about 40 sec was obtained corresponding to a "half time" of 28 sec which is in agreement with previous observations (Henry and De Moor 1956 Margaria *et al.* 1965 Cerretelli *et al.* 1966 III Prampero *et al.* 1970 Whipp 1971 Whipp and Wasserman 1972). The corresponding value for $\dot{V}O_{2(PC)}$ was 5–10 sec shorter indicating that some 0.1 l O_2 was drawn from the pulmonary O_2 stores at the start of work and then repaid during recovery. In subjects with a sluggish \dot{V}_E response the changes in pulmonary O_2 stores may be greater and it may then not be possible to discern two phases of the $\dot{V}O_{2(PC)}$ response from breath-by-breath recordings of $\dot{V}O_2$.

During heavy and very heavy work there was a continuous slow change in $\dot{V}O_2$ (*third phase*) also after the second min of exercise (Fig. 7). With these work loads the $\dot{V}O_2$ responses could be described as second-order functions with one short time constant describing the first two phases of the response (rapid components) and one much longer time constant describing the slow component (Table IV). The short time constant was

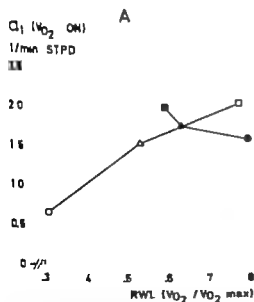
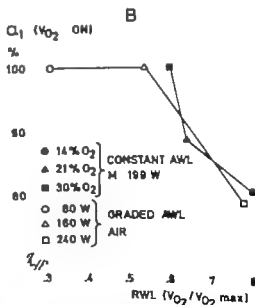


Fig. 18 A Amplitude (a_1) of the short time constant of the $\dot{V}O_2$ on-response (*cf* Table IV) as a function of the relative work load (RWL). Note that a_1 increases with the absolute work load (AWL) but decreases for constant AWL when RWL is increased by hypoxia. For symbols, see B.



B a_1 in per cent of the O_2 need (*cf* § 26) as a function of RWL. Note that for RWL values exceeding about 0.6 the relative contribution of the rapid time constant was progressively reduced both with increasing AWL values and when PiO_2 was lowered at a constant AWL, averaging 199 W.

the discrepancy between the metabolic need and the O_2 delivery the resulting, gradually developing lactate accumulation or related metabolic change providing an "error signal" for a further slow adjustment in the O_2 transport

The aforementioned observation that $\dot{V}O_2$ during the 6th min of exercise was lower in hypoxia than in air indicates that in the former condition no steady state level of O_2 delivery was attained. In contrast under experimental conditions where a true steady state can be obtained during exercise in hypoxia heart rate and cardiac output have been shown to increase sufficiently to compensate for a reduced arterial O_2 content so that the arterial transport of O_2 equals that during normoxia (Hughes *et al.* 1968)

The dynamic behavior of $\dot{V}O_2$ in response to on and off-changes in work load may be expressed in terms of O_2 deficit and O_2 debt. These parameters reflect the overall rate of response and also in what extent steady-state levels of $\dot{V}O_2$ are attained. In Fig. 19 O_2 deficit and O_2 debt both for the graded and the constant AWL experiments have been related to the relative work level (cf Knuttgen 1970 Knuttgen and Saltin 1973). The O_2 deficit increased both with the absolute and the relative work load. The O_2 debt also increased with AWL and RWL, and debt and deficit values agreed closely except in hypoxia where the large deficit was accompanied by a lower O_2 debt. The observation that the

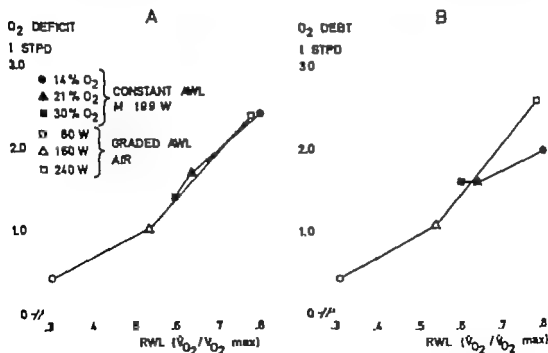


Fig. 19 A O_2 deficit as a function of relative work load.

B O_2 debt as a function of relative work load, for symbols, see A

debt/deficit ratio was equal to or less than unity with heavy and very heavy work differs from the classical concept that the repayment of the lactic O_2 debt occurring after work demanding more than $2/3$ of $\dot{V}O_{2\max}$ (Margarita Edwards and Dill 1933) is associated with a debt/deficit ratio greater than unity (Asmussen 1946 Christensen and Högberg 1950, Henry and De Moor 1950). A tentative explanation for this discrepancy in results may be found in the fact that in the present experiments light work (0-load) was performed during the recovery periods which would allow lactate to be oxidized as a substrate (Jorfeldt 1970 Hermansen and Stensvold 1972). This way of eliminating lactate would be associated with no extra O_2 cost in contrast to gluconeogenesis which is an energy demanding route of lactate elimination. The magnitude of the O_2 debt is thus influenced by the relative contributions of these two metabolic pathways for lactate elimination (cf Whipp Seard and Wasserman 1970). Furthermore the ability to oxidize lactate as a substrate has been shown to be enhanced during a given work period when preceded by prolonged heavy exercise (Karlsson 1971). This may have influenced the pathway of lactate elimination and hence the O_2 debt particularly in case of the present hypoxic experiments, where the relative work load was higher than in any other experimental condition and furthermore the work was performed repeatedly.

Dynamic Behavior of $\dot{V}CO_2$ and R in Response to Step Changes in Work Load

The overall responses of $\dot{V}CO_2$ were in all conditions considerably slower than those of $\dot{V}O_2$. This is in agreement with previous observations by Robinson (1938) Cerretelli *et al* (1966) and Edwards (1969). The tissue stores for CO_2 appreciably exceed those for O_2 (Farhi and Rahn 1955) and it has been suggested that the buffering action of large peripheral CO_2 stores explains why changes in mixed venous PCO_2 occur less rapidly than the corresponding PO_2 changes during onset of exercise (Edwards *et al* 1972). Until the time when blood of altered composition due to a change in work load reaches the lungs the changes in $\dot{V}CO_2$ will depend mainly on changes in cardiac output in the same way as $\dot{V}O_2$ and $\dot{V}O_{2(FC)}$. Accordingly R exhibited only minor changes during the initial 15–20 sec period after a change in work load and then showed a transient drop during exercise (cf Davies 1968), and a transient overshoot during the first minutes of recovery (Fig. 7 10). The more pronounced overshoot during recovery from very heavy exercise (cf Hansen 1934) indicates that CO_2 is driven off from the body in excess of the metabolic production due to the accumulation of fixed acids, notably lactate.

VI GENERAL SUMMARY

The time courses of O_2 uptake pulmonary-capillary O_2 transfer ($\dot{V}O_{2(PC)}$) and ventilatory functions at start and end of exercise were studied on a breath-by-breath basis together with that of the heart rate. Responses to step changes in work load formed the basis for the subsequent identification and quantitation of time components using a computer-oriented technique. Experiments were carried out on 8 subjects performing light (80 W) moderate (160 W) and very heavy exercise (240 W) on a cycle ergometer in the sitting position breathing air and with zero-load pedalling serving as the base line. In these experiments changes in the dynamic behavior of pulmonary gas exchange and heart rate were studied with respect to the effects of variations in absolute work load. In a second series of experiments a heavy work load was used constant for each subject during hyperoxia (30 % O_2) normoxia (air) and hypoxia (14 % O_2) corresponding to relative work intensities of 60 64 and 80 per cent respectively of the maximal aerobic capacity. By comparing the results from the two studies the recorded time courses could be related both to the absolute and to the relative work loads.

Dynamics of O_2 Uptake in Response to Light and Moderate Work

The adjustments of $\dot{V}O_{2(PC)}$ during light and moderate work were completed within 2 min after the start and end of exercise and the time courses could be described as monoexponential functions with a time constant approximating 35 sec for both the on- and off responses in the two conditions. Close inspection of individual and group mean responses revealed however that a more precise description of the $\dot{V}O_{2(PC)}$ response should include two phases: (a) a rapid initial change during the first 15–20 sec followed by (b) a second rapid rise lasting until about 1.5–2 min had elapsed after the change in work load. The first phase which presumably resulted from rapid changes in blood flow through the lungs, accounted in the on-responses for 50 and 35 per cent of the final $\dot{V}O_{2(PC)}$ change during light and moderate work, respectively. The second phase presumably reflected changes not only in cardiac output but also in the arteriovenous O_2 difference the latter change appearing in the lungs only after a 15–20 sec delay corresponding to the time required for the transport of blood from the working muscles. This interpretation is supported by the observation of a relative constancy of O_2 pulse end-expired PO_2 and PCO_2 and respiratory exchange ratio during the initial 15–20 sec period after a change in work load.

The responses of $\dot{V}O$ were in general slower than those of $\dot{V}O_{2(PC)}$ during the first

15–20 sec following a change in work load but thereafter the two time courses agreed closely. Thus the overall rates of response expressed as mean response times (MRT) were 5–10 sec longer for \dot{V}_{O_2} than for $\dot{V}_{O_2(FC)}$ indicating that some 0.1 l O_2 was drawn from the pulmonary O_2 stores at start of exercise and then repaid during recovery.

Dynamics of O_2 Uptake in Response to Heavy and Very Heavy Work

The basic step responses of $\dot{V}_{O_2(FC)}$ with a time constant of about 35 sec were present also during unsteady state of very heavy work. However the magnitude of the rapid changes during the initial phases of the on responses was not sufficient to meet the need for O_2 and consequently $\dot{V}_{O_2(FC)}$ continued to increase slowly throughout the 6-min work period. The $\dot{V}_{O_2(FC)}$ responses in very heavy exercise could thus be better described as second-order functions the shorter time constant accounting for about 80 per cent of the final 6th min response. Also during heavy work in air and in hypoxia the appearance of a slow component required second-order models to describe the $\dot{V}_{O_2(FC)}$ responses whereas in hyperoxia the $\dot{V}_{O_2(FC)}$ response to the same heavy work load could be described as a first-order function. Thus, an increase in relative work load (RWL) was accompanied by similar changes in $\dot{V}_{O_2(FC)}$ response regardless of whether the increase in RWL was effected by increasing the absolute work load or by the inhalation of a hypoxic gas mixture at a constant preset load. This indicates that the time course of $\dot{V}_{O_2(FC)}$ is determined by the rate of acceleration of the circulatory O_2 transport and not by any inertia of the peripheral processes of O_2 utilization.

A similarity between on- and off-responses of O_2 uptake was observed also in heavy and very heavy work, except during hypoxia. As a consequence O_2 -debt and O_2 -deficit values agreed closely in any given condition except in hypoxia where the O_2 debt was slightly lower than the O_2 deficit.

Dynamics of Ventilation and Heart Rate Changes

The ventilatory on- and off-responses were both found to consist of two distinctly different phases. The first phase of the on response was primarily due to a rapid increase in tidal volume brought about by a reduction of the functional residual capacity the second and slower phase occurring with a delay of 15–20 sec, i.e. at the time when blood from the working muscles can be assumed to have reached arterial or medullary chemoreceptors. The overall rate of change of \dot{V}_I at the onset of work differed only slightly between the various conditions with MRT values approximating 70 sec. During recovery however

the rate of change of V_E was markedly slowed with increasing absolute work load where-
as no such slowing occurred when RWL was increased by hypoxia.

In general the overall heart rate responses were more rapid than the corresponding ventilatory responses. At the onset of work HR showed a very rapid initial increase followed by a short-lasting decrease or plateau before a second rise occurred. With higher work loads the heart rate like V_{O_2} showed a slow component in both the on- and off responses which could be described by second-order models. The response of HR at the start of exercise seemed to depend on the absolute work load the MRT values ranging from 15 sec during light work to 40 sec during very heavy work. During recovery the HR responses generally were slower than the corresponding on responses and became slower the higher the relative work load.

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ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 416

AFTERHYPERPOLARIZATION AND THE
CONTROL OF REPETITIVE FIRING IN
SPINAL NEURONES OF THE CAT

BY
BENGT GUSTAFSSON

GÖTEBORG 1974

RESULTS AND COMMENTS

I Afterhyperpolarization properties

A AHP conductance time course

1 α -motoneurons The AHP recorded in α -motoneurons lasts 40-200 ms and reaches a peak value 10-20 ms after the onset of the action potential (Brock et al 1952 Eccles et al 1958 Kuno 1959). Usually the rapid falling phase of the spike is not immediately followed by the AHP but is succeeded by a phase of slow repolarization lasting for some ms, the delayed depolarization (DD; Kernell 1964 Nelson and Burke 1967). In some motoneurons a clear depolarizing potential can be seen in this phase giving a "hump-type-DD".

From the effect of membrane potential displacements and of iontophoresis on the AHP voltage (Coombs, Eccles and Fatt 1955) it was concluded that the AHP in motoneurons is mainly resulting from a membrane conductance increase to potassium ions. The time course of the underlying conductance process was however not deduced. It has thus not been clear to which extent the long time-to-peak AHP was given by the conductance process as suggested by Eccles (1957) or by the process giving rise to the DD (Kernell and Sjöholm 1972). The origin of this latter process is also unknown, but it has been suggested to be caused by current fed back from the spike invasion into the dendrites (Granit et al 1963 Kernell 1964 Nelson and Burke 1967) or by an unspecific conductance change (mainly sodium; Kernell and Sjöholm 1972).

In Paper I the conductance time course during the AHP was determined by the application of short current pulses injected through the recording microelectrode. The time course so measured, displayed an initial fast decay during the rapid hyperpolarizing phase of the AHP followed by a phase of constant or slightly increasing (or decreasing) conductance lasting to the peak of the AHP. Thereafter the conductance decreased in a rather exponential manner during the falling phase of the AHP. A similar conductance time course was also observed during the AHP in the presence of the DD. The experimental ones, by showing a dip in the conductance with the DD, was also absent.

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FROM THE DEPARTMENT OF PHYSIOLOGY UNIVERSITY OF GÖTEBORG, SWEDEN

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CONTROL OF REPETITIVE FIRING IN
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This thesis mainly constitutes a summary of the following articles

- I Baldissera, F and B Gustafsson, Afterhyperpolarization conductance time course in lumbar motoneurons of the cat. *Acta physiol scand* 1974 In press
- II Baldissera, F and B Gustafsson, Firing behaviour of a neurone model based on the afterhyperpolarization conductance time course First interval firing *Acta physiol scand* 1974 In press
- III Baldissera, F and B Gustafsson Firing behaviour of a neurone model based on the afterhyperpolarization conductance time course and algebraical summation. Adaptation and steady state firing *Acta physiol scand* 1974 In press
- IV Gustafsson, B S Lindström and M Takata A re-evaluation of the afterhyperpolarization mechanism in dorsal spinocerebellar tract neurons *Brain Res* 1971 35 643-646
- V Gustafsson, B S Lindström and M Takata Repetitive firing in dorsal spinocerebellar tract neurones *Brain Res* 1972 47 506-509
- VI Gustafsson B and P Zangger Depression of the afterhyperpolarization in dorsal spinocerebellar tract neurones *Brain Res* 1974 72 320-323

These papers will be referred to by their Roman numerals in the text.

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INTRODUCTION

In the nervous system information carried between nerve cells is encoded in the frequency of action potentials transmitted along the axons. The steady excitation built up by the continuous synaptic activity impinging upon a neurone is thus converted into a discontinuous output i.e. a train of action potentials whose frequency is related to the amount of excitation. The encoding procedure, i.e. the mechanism controlling the relation between excitation and frequency was early visualized by Adrian (Adrian and Zotterman 1926) as follows. After the neurone (or receptor) has fired an action potential, the neurone is in a refractory state, i.e. the threshold for giving an action potential is increased. The threshold increase is then slowly decaying with a certain time course. With a just suprathreshold stimulation the interval between the impulses will then be equal to the duration of the refractoriness. Increasing the excitation the refractoriness will be overcome at an earlier interval i.e. the frequency of action potentials will increase. There will thus be a direct correspondence between the amount of excitation and the frequency of action potentials and this relation will be completely determined by the size and shape of the time course of the refractoriness. When studied with intracellular technique the nerve cells were in fact, found to possess a longlasting refractoriness following each action potential. In α -motoneurons in the cat spinal cord the action potential was followed by an afterhyperpolarization (AHP) lasting 40-200 ms (Brock, Coombs and Eccles 1952; Eccles, Eccles and Lundberg 1958; Kuno 1959).

The hypothesis of Adrian could then be tested in these neurones by comparing the time course of the refractoriness given by the AHP with the relation between excitatory current and impulse frequency as outlined above. The technique used consists in injecting the excitatory current as constant current pulses from an intracellular micro-electrode and measuring the discharge frequency at various current strengths (Granit, Kornell and Smith 1963; Granit, Kornell and Shortness 1963 a, b). In such investigations (Kornell 1965 b & d) it was demonstrated that the minimal rhythmic frequency was correlated with the duration of the AHP as predicted by Adrian, and also other correlations between AHP parameters and the firing were found. However, due to the lack of sufficient knowledge of the mechanism underlying the AHP in motoneurons, more critical tests aimed at determining to what extent the firing was regulated by the AHP in the manner described by Adrian could not be made and certain findings were even indicating that other mechanisms could be of importance (Kornell 1965 c; Granit, Kornell and Lamarre 1966 b). It was understood that if the encoding should be

only determined by the AHP time course, the afterpotential mechanism must be invariant of all other activity in the cell. If instead the amplitude of the afterpotential for example was a function of the degree of the antidromic invasion of the nerve impulse into the dendritic tree and if this invasion could be affected by various factors then these factors could also be of considerable importance for the firing regulation. Even if this view still pointed to the importance of refractoriness for firing regulation it precluded the possibility to predict firing behaviour from the shape of the refractoriness as measured after single action potentials. This alternative hypothesis originated in the finding that in many motoneurons the frequency-current (f/I) relation at higher frequencies suddenly bent upwards and displayed a second linear range (secondary range) whose characteristics could be modified by synaptic activity and whose appearance was correlated with a sudden change in the interspike voltage course implying a change in the AHP properties (Kernell 1965 c; Granit et al. 1966 b). This hypothesis was later expanded by Schwindt and Calvin (1972) who suggested that more or less continuous changes in the spatial distribution of the AHP could be a factor of importance for the input-output relation in motoneurons.

There are, however, some evidence that the above alternative hypothesis is disputable. Recently Kernell and Sjöholm (1973) demonstrated that by modifying voltage clamp equations from the myelinated frog nerve fibre to give a longlasting AHP some of the features of the firing behaviour of motoneurons previously indicative of a spatial factor could be simulated. However, even if this model rather well simulated the f/I relations in motoneurons it did not well simulate the characteristic modifications of the interspike voltage trajectories with increasing current described for real motoneurons (Schwindt and Calvin 1972, Schwindt 1973). Moreover, it is not clear to which extent the mechanism underlying the AHP in this model is similar to that in motoneurons.

The α -motoneurons have in many respects been used as a model neuron for central neurons. The firing behaviour of the few other neurons investigated was, however, found to differ from that of α -motoneurons to some extent (Kandel and Spencer 1961; Kuno and Miyahara 1968; Elde, Fedina, Jansen, Lundberg and Vyklický 1969; Kofke, Mano, Okada and Oshima 1970; Baker and Precht 1972). For example, in the cells belonging to the dorsal spinocerebellar tract (DSCT) the f/I relation is linear throughout its frequency range without displaying a 'secondary range' as in motoneurons and has a much steeper slope than in motoneurons (Elde et al. 1969).

Two different suggestions have been forwarded to explain this difference. According to one of these, the absence of a secondary range could be accounted for by a lack of change in dendritic invasion

with increasing current, thus implicating the importance of spatial factors in firing regulation (Eide et al 1969). The other hypothesis pointed instead to the possibility that the AHP mechanism in DSCT neurones is of a different nature than that of motoneurones - a suggestion which has also received some experimental support (Eide et al 1969, Kuno, Miyahara and Weakly 1970).

AIM OF THE PRESENT STUDY

Even if it is generally agreed that the AHP is a factor of considerable importance for the firing regulation in central neurones the exact role of it and of other factors are still uncertain or as recently drastically stated (Schwindt and Calvin 1973 b) 'the mechanisms underlying rhythmic firing in motoneurones are virtually unknown - a statement then referable to other central neurones as well. This statement is in one sense justified as long as no direct correlation between the AHP properties and the firing behaviour is made, outlining the role of the AHP and the necessity and possible role of other factors

The aim of the present study is to demonstrate to which extent the AHP can account for the firing behaviour in two different types of spinal neurones - the α -motoneurones and the DSCT neurones. The study will cover two sections. The first section will deal with investigations on the AHP mechanism in motoneurones and DSCT neurones in order to deduce the nature and the time course of the underlying processes. In the second section the firing behaviour predicted by a model based on these processes will be computed and compared to the firing behaviour of the real neurones.

RESULTS AND COMMENTS

I Afterhyperpolarization properties

A AHP conductance time course

1 α -motoneurons The AHP recorded in α -motoneurons lasts 40-200 ms and reaches a peak value 10-20 ms after the onset of the action potential (Brock et al. 1953; Eccles et al. 1958; Kuno 1959). Usually the rapid falling phase of the spike is not immediately followed by the AHP but is succeeded by a phase of slow repolarization lasting for some ms, the delayed depolarization (DD; Kernell 1964; Nelson and Burke 1967). In some motoneurons a clear depolarizing potential can be seen in this phase giving a "hump-type-DD".

From the effect of membrane potential displacements and of iontophoresis on the AHP voltage (Coombs, Eccles and Fatt 1955) it was concluded that the AHP in motoneurons is mainly resulting from a membrane conductance increase to potassium ions. The time course of the underlying conductance process was however not deduced. It has thus not been clear to which extent the long time-to-peak AHP was given by the conductance process as suggested by Eccles (1957) or by the process giving rise to the DD (Kernell and Sjöholm 1972). The origin of this latter process is also unknown, but it has been suggested to be caused by current fed back from the spike invasion into the dendrites (Granit et al. 1963; Kernell 1964; Nelson and Burke 1967) or by an unspecific conductance change (mainly sodium; Kernell and Sjöholm 1972).

In Paper I the conductance time course during the AHP was determined by the application of short current pulses injected through the recording microelectrode. The time course so measured, displayed an initial fast decay during the rapid hyperpolarizing phase of the AHP followed by a phase of constant or slightly increasing (or decreasing) conductance lasting to the peak of the AHP. Thereafter the conductance decreased in a rather exponential manner during the repolarizing phase of the AHP. A similar conductance time course was also computed from the AHP voltage, under the assumption that the AHP was only given by a K conductance increase. The computed time courses differed, from the experimental ones by showing a shortlasting dip in the fast initial decay phase. This dip was found to be correlated with the size of the DD and was also absent in neurones lacking an apparent DD. It was concluded

that the AHP in motoneurons was given by a conductance time course as suggested by Eccles (1957) and that the DD was given by a short-lasting current, not giving rise to the long time-to-peak AHP

It was assumed by Coombs et al (1955) that the mechanism underlying the AHP was not much affected by the membrane potential. This assumption was largely corroborated in the present study by showing a constancy both in the voltage drop caused by a current pulse evoked during the AHP at different membrane potential levels and in the computed conductance time course at different potential levels. This conclusion was of considerable significance since it meant that the AHP potassium conductance could be described only as a function of time and not as a function of both voltage and time. It was concluded that the AHP in α -motoneurons was given by a complex function in time describing an initial fast decay, a plateau region lasting to the peak of the AHP and a slow exponential decay. A mathematical function describing this time course was then derived.

2 DSCT neurones Previous investigations (Kuno and Miyahara 1968, Eide et al 1969, Kuno et al, 1970) had shown the AHP in DSCT neurones to be appreciably smaller and shorter than that in motoneurons. As stated in the Introduction these investigations had also given evidence that the AHP in DSCT neurones was not caused by a K conductance mechanism as in α -motoneurons. Kuno et al. (1970) had found that there was no conductance increase during the AHP, that the AHP voltage was not potential dependent, that the AHP polarised the membrane to more negative levels than the potassium equilibrium potential and that the AHP had a temperature dependence incompatible with a passive ionic process. They concluded that the AHP instead was caused by the activation of a sodium electrogenic pump. On the other hand, Eide et al. (1969) found the AHP voltage to be very sensitive to polarising currents but found only small, if any, conductance changes. They suggested that the AHP at least partly could be caused by a sodium conductance decrease. The apparent discrepancies in the reported results made a complete reinvestigation of the AHP properties in DSCT neurones necessary.

In such a reinvestigation of the AHP properties in DSCT neurones (Paper IV) it was demonstrated that the AHP in these cells as in motoneurons is caused by a conductance process for potassium ions. By displacement of the membrane potential with current injections the AHP voltage was found to increase with depolarization and decrease by hyperpolarization, as found by Eide et al (1969) and as expected for a conductance process. The conductance during the AHP was measured by application of current pulses and a clear conductance increase was found in all the cells tested. By applying the same pulses during another

hyperpolarizing pulse, it was established that the measured conductance changes were not secondary to the hyperpolarization itself. These findings indicated that the AHP was neither given by an electrogenic pump nor by a sodium conductance decrease process. However, if the pump or the sodium conductance were potential dependent, increasing with depolarization and decreasing with hyperpolarization, similar experimental results could, in fact, also be produced by these mechanisms. In a few neurones the AHP could, however, be reversed and current pulses applied during these reversed AHPs demonstrated a conductance increase (Gustafsson, Lindström and Takata, to be published). Since these experimental results could not be given by the two former mechanisms, it can be concluded that the AHP in DSCT neurones is given by a K conductance mechanism.

It had further been argued by Kuno et al. (1970) that the AHP could not be given by a K conductance process since the AHP polarized the membrane to more negative levels than the K equilibrium potential and the AHP decay had a temperature dependence incompatible with a passive ionic process. The last objection was not relevant since the temperature dependence of the decay of passive ionic processes, in contrast to that of the amplitude, is rather similar to that of active metabolic processes (Hodgkin and Huxley 1952). This point is made very clear by the recent finding that there is a temperature dependence of the AHP decay also in motoneurones (Pierau, Klee and Faber 1971). The objection that the AHP polarized the membrane to more negative values than the potassium equilibrium potential was also easily ruled out since Kuno et al. (1970) had not estimated any potassium equilibrium potential. In nerve cells the potassium equilibrium potential has only been estimated from the reversal potential of the AHP. Since Kuno et al. did not find any potential dependence of the AHP, no reversal potential and thus K equilibrium potential could be estimated. The equilibrium potential was instead taken as the "reversal potential of the initial overruling immediately following the falling phase of the spike". However, due to the presence of a delayed depolarization in this region, this "reversal potential is not equal to the K equilibrium potential".

Earlier investigations (Kuno and Miyahara 1968, Eide et al. 1969, Kuno et al. 1970) had found the AHP in DSCT neurones to be smaller and shorter than that of motoneurones. In the present study the computed time course of the conductance underlying the AHP in DSCT neurones was also found to be different from that in motoneurones, displaying no or only a small plateau phase before the subsequent exponential decay (see Fig. 1). This time course was also reflected in a considerable shorter time-to-peak AHP, being only on the average 7.5 ms in DSCT neurones (Gustafsson, Lindström and Takata, to be published) compared to 10-20 ms in motoneurones. Otherwise the computed $G_K(t)$ had a similar

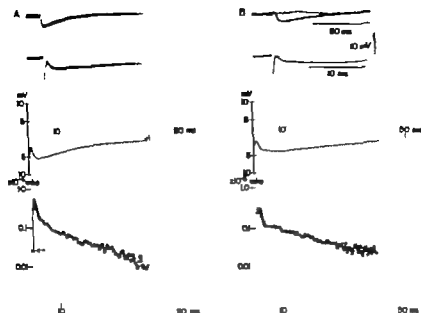


Fig 1 Computed AHP conductance time course from two DSCT neurones. The conductance time courses computed from the AHP voltage are shown for two DSCT neurones with a short time-to-peak AHP (A) and a long time-to-peak AHP (B). Records from which the measurements were taken are shown above each graph. Observe the small plateau in the computed capacitance in B corresponding to the slow hyperpolarizing phase of the AHP. The initial dip given by the DD is marked in A with an arrow. Input resistance ($M\Omega$) membrane capacitance (nF 10^{-9} F) are 2.20 2.85 (A) 2.90 3.60 (B). AHP equilibrium potential for both cells assumed to be 30 mV below resting level.

course to that in motoneurones. I.e. there was an initial dip corresponding to the DD, followed by an initial fast decay during the hyperpolarizing phase of the AHP and a slow exponential decay during the repolarizing phase of the AHP. A similar conductance time course was also found in one DSCT neurone with the use of short constant current pulses (Gustafsson, Lindström and Tekata, to be published).

In some central neurones including the α -motoneurones and the DSCT neurones the AHP following a short train of spikes is larger than that following a single spike i.e. the successive AHPs summate (Ito and Oshima 1962 Elde et al. 1969 Baker and Precht 1972 Kitai, Tanaka Tsukahara and Yu 1972). Since this summation has been considered as a mechanism for the decrease in frequency (adaptation) occurring in neurones after the onset of a constant current pulse (Kernell 1968 1972 Calvin and Schwindt 1972) knowledge of its properties should be of importance for the understanding of firing regulation. This summation effect is largest at short interspike intervals and decays to zero when the interspike interval approaches the duration of the AHP. In α -motoneurones the time course of the summation of the AHP voltage between two successive AHPs was not unlike the time course of the AHP itself (Calvin and Schwindt 1972). In the present investigation the conductance summation between two successive AHPs was studied (Paper III) and a similar conclusion was reached. The increase in the AHP peak conductance (computed and estimated with short current pulses) of the second AHP was following a course rather similar to that of the conductance of the first AHP i.e. there seemed to be an algebraical summation between the two AHPs. There was however in most of the cells studied a quantitative deviation from the algebraical summation, the absolute summation being both larger and smaller.

In DSCT neurones the AHP interaction had previously only been studied with short interspike intervals between the action potentials and an AHP summation had been found (Elde et al. 1969 Kuno et al. 1970). At longer interspike intervals the AHP interaction was however more complex than in motoneurones (Paper VI). In the majority of the cells the AHP following two spikes was in fact, smaller than that following a single spike the depression being maximal at an interval corresponding to the AHP duration. The falling phase and the initial hyperpolarizing overshwing of the spike was unaffected by the conditioning spike demonstrating a separation of the K conductance underlying the AHP and that giving the falling phase of the action potential. The AHP voltage from the peak of the DD was symmetrically decreased and subsequent conductance computation demonstrated the depression to symmetrically affect the whole computed time course as described earlier i.e. both the first fast decay and the subsequent slow decay (Gustafsson and Zangger to be published). The finding that the neurones with large depression also had a smaller AHP summation at short intervals suggested that the depression and summation, in fact, were both present throughout the interspike interval. An interesting observation was here that, in the neurones with large depression the hyperpolarizing phase of the AHP following a single spike was faster than that

following two spikes in quick succession in spite of an AHP summation. This difference in AHP time course can however be explained as a consequence of the depression, being caused by the linear summation of the conductance remaining from the first spike to the depressed conductance from the second spike (Gustafsson and Zangger to be published).

The depressive effect was not accumulating the successive AHPs in a long train (with an interval = AHP duration) being of the same size as the second one. No apparent increase in depression was found when conditioning a single spike with a train of spikes instead of with a single spike (Gustafsson and Zangger to be published).

Otherwise the AHP interaction in DSCT neurones behaved very similar to that given by a linear summation. The final value of the AHP summation was reached in a rather exponential fashion, the slope and peak value increasing with decreasing interval. There was no change in the time constant of AHP decay (computed time course) between AHPs following a single spike and a train of 15 spikes (Gustafsson and Zangger to be published).

II Repetitive firing of a neurone model based on the AHP conductance time course compared to that of real neurones

The aim of the present investigations was to study to which extent the firing behaviour of motoneurones and DSCT neurones is accounted for by the AHP properties. From the results presented in the preceding section and with some simplifying assumptions of the neuronal properties a neurone model for repetitive firing based on the AHP properties can be formulated as follows:

- 1 The K conductance underlying the AHP is potential independent and has a time course as described in the preceding section (see Paper I and Fig. 2). Its equilibrium potential is constant.

- 2 The K conductance evoked by one spike is unaffected by the succeeding spikes.

- 3 The K conductance evoked by the second and following spikes after the onset of a constant current can be multiplied with a correction factor in respect to that evoked by the first spike.

- 4 The delayed depolarisation is given by a shortlasting depolarising current pulse, which is constant for each spike (see Fig. 3).

5 All other conductances but the K conductance are constant throughout the interspike interval and their equilibrium potentials are constant

6 The threshold for spike activation is constant, the spike duration is constant and the falling phase of the spike ends at a constant voltage level

7 The complex geometry of the neurone can be neglected and the neurone can be represented as a sphere

Before entering the computed and experimental firing behaviour demonstrated in the present investigation previous results on the repetitive firing in motoneurones and DSCT neurones and the descriptive terminology developed in these investigations will be reviewed

In most α -motoneurones and DSCT neurones the application of a constant current pulse gives rise to a repetitive discharge, whose frequency is dependent upon the current strength. After the onset of the current pulse there is a rapid decrease in frequency (adaptation) which is usually completed within the first few spikes but may continue for a longer time (Kernell 1968 & Eide et al 1969)

In α -motoneurones the frequency-current (f/I) relation and interspike voltage trajectory behaviour has mostly been studied after adaptation is completed, i.e. at the steady state. The steady state f/I relation is composed of three linear segments, the 'primary', 'secondary' and 'tertiary' range (Kernell 1968 & Schwindt 1973). The 'primary' range extends from the minimal rhythmic frequency up to a maximal frequency corresponding to 1/time-to-peak AHP followed, in some motoneurones by a second, linear steeper segment, the 'secondary' range (Kernell 1968 & c). In other motoneurones the continuous firing failed before reaching higher frequencies and only a 'primary' range was found. A third linear segment, the 'tertiary' range, with a slope usually less than that in the 'secondary' range, has been found in some motoneurones (Schwindt 1973).

In the trajectory analysis (Schwindt and Calvin 1972, Schwindt 1973) the AHP voltage was subdivided in two segments: the 'scoop' being the descending and bottoming portion of the AHP and the 'ramp' being the subsequent repolarization to firing level. It was suggested that the different firing ranges were associated with a special trajectory behaviour. In the 'primary' range the trajectory was characterised by a 'scoop-change' behaviour, i.e. with increasing current the size of the scoop was diminished while the ramp slope was constant (or stereotyped). In the 'secondary' range the scoop had an upward convex shape instead of the upward concave shape found in the 'primary' range. The firing was in the 'secondary' range controlled by a 'ramp change' behaviour, i.e. the

firing level was now reached by changing the slope of the trajectory. The entrance into the tertiary range was associated with a sudden change in trajectory shape and the interspike trajectory assumed an angular form with a downward peak (Schwindt 1973). Within the tertiary range there was again a scoop-change behaviour i.e. with increasing current the peak was decreased while the slope of the ramp remained constant. A similar f/i and trajectory behaviour was also described for the first interspike intervals i.e. before adaptation.

In the DSOT neurones the steady state f/i relation was found to exhibit only one linear segment, reaching up to very high frequencies (Eide et al. 1969). For the first interspike intervals i.e. before adaptation, only the higher frequency range was investigated, displaying an upward concave relation (Kuno and Miyahara 1968). No analysis of the trajectories was done in these studies.

The computation of the firing behaviour of the neurone model was first performed without the assumption 2, i.e. for a model without AHP "summation". This would then be similar to the first interspike interval after the current onset in real neurones.

A First interspike interval computation

1 Computation with conductance plateau. With this "motoneurone-like" model most of the features already described from motoneurones could be found (Paper II). With increasing current the upward concave scoop first flattened and then assumed an upward convex shape. At very high currents the trajectory assumed the downward angular shape described for real neurones. The f/i relation also showed three more or less distinct segments: Below the frequency corresponding to 1/time-to-peak AHP the f/i slope started to increase considerably i.e. the f/i relation was displaying a secondary range. At still higher currents the f/i slope decreased again and assumed an upward convex shape. There were, however, some clear differences between the model behaviour and the behaviour of real neurones as described earlier. In the model, the ramp slope was not linear at the very low frequencies but displayed a slightly upward convex shape. The approximated ramp slope was not constant but increased with increasing current. However, by scrutiny of the records published by Schwindt and Calvin (1972) it was clear that the model behaviour was not in contrast to their results. While Schwindt and Calvin emphasized the linear and the stereotyped character of the ramp the real motoneurone trajectories were in fact also upward convex in shape at the very low frequencies and the ramp slope at increasing current could, in fact, also be force fitted by steeper lines in much the same way as in the model (see also Paper III).

The flattening and the subsequent "reversal" of the scoop while gradual, was earlier described (Schwindt and Calvin 1972) as taking place in connexion with the entrance into the 'secondary range of firing. In the model this was not the case; the scoop change occurring already well within the 'primary range, the exact timing depending upon the shape of the $G_K(t)$ plateau.

Since the previous studies were mostly concerned with the steady state firing the relation between the first interval trajectory modifications and the f/I shape was investigated and the behaviour displayed by the model was in fact also found in the motoneurones examined.

It was earlier described that a hump-type delayed depolarization seemed to appear in some motoneurones in connexion with the entrance into the 'secondary range (Schwindt and Calvin 1972). The published records indicated, however, that this "DD" was appreciably later than the DD found at resting level and suggested to us that it instead was coupled with the $G_K(t)$ local minimum (see Paper D). This later hump should then be preceded in real motoneurones by a DD and such a double-hump trajectory sequence was also found in real motoneurones.

The rise to firing level during the 'primary range was earlier described as being stereotyped (Schwindt and Calvin 1972) while during secondary range the trajectory slope was increasing with increasing current (Schwindt 1973). Also in the model the rate of rise to firing level increases with increasing current during the secondary range more than during the 'primary range of firing. This trajectory behaviour during secondary range is however not related to entering the secondary range but is a continuation of the scoop rotation around the pivot point given by the falling phase of the spike present also all through the 'primary range.

The entrance into the 'tertiary range of firing was earlier described (Schwindt 1973) to occur together with a sudden change in trajectory shape, indicative of a sudden change in cell properties. This sudden change is also reproduced by the model and is occurring when the scoop is fully rotated and thus the $G_K(t)$ plateau region passed. The trajectory will then assume the simple downward angular shape in the model as in real neurones (Schwindt 1973).

The model behaviour gave also an additional prediction of firing behaviour. When computing the first interval firing with a pronounced $G_K(t)$ local minimum no interval values corresponding to the region surrounding the local maximum were found, i.e. there was a discontinuity in the f/I relation, not earlier described for motoneurones. Such a jump in the first interval f/I relation was however also displayed by some of the motoneurones examined, thus giving a discontinuity.

ity between the upper part of the 'primary' range and the lower end of the upper convex part of the f/I relation or 'tertiary' range

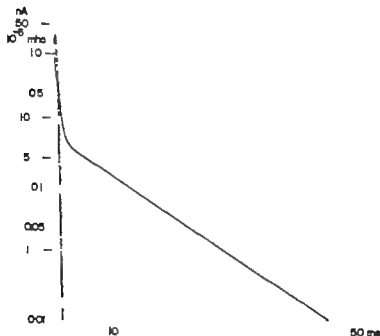
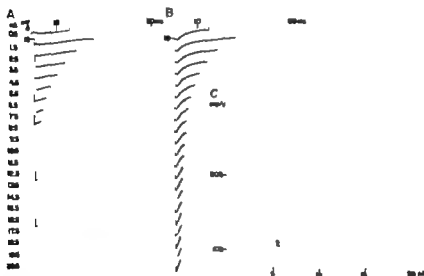


Fig. 2 Graphic representation of the equations representing the AHP K conductance and DD current in DSCT neurones. Dotted line is representing the DD current and is given by the equation $60 \times \text{EXP}(-t/2) \times 10^{-6}$ expressed in nA. The continuous line is representing the AHP K conductance and is given by the equation $0.25 \times \text{EXP}(-t/14) + 1.1 \times \text{EXP}(-t/2)$ expressed in 10^{-6} mho.

2 Computation without a conductance plateau
 The trajectories resulting from this "DSCT" like model could also be subdivided into a scoop and a ramp segment (see Fig 3). The shape of these trajectories and their modification with increasing current injection was however somewhat different from that of the "motor-neurone" ("Mn") model. Moreover the trajectory shape was also much dependent upon the size of the DD (or the magnitude of the initial fast $G_K(t)$ decay). With a DD present (Fig 3 A) the scoop started at the peak of the DD and reached an early peak, followed by an almost linear slightly upward convex ramp. With increasing current the scoop decreased successively while the ramp slope increased. This pattern of trajectory modification continued up to very short interspike intervals (note the upward concavity still at 11.5 nA in Fig 3 A) until the firing started at the peak of the DD and the trajectory assumed a downward angular shape. At all current strengths the DD gave an



initial upward convexity not to be confused with the upward convexity given by the $G_K(t)$ plateau, lacking in this model version. The f/I relation given by these trajectories (filled circles in C) was also somewhat different from that of the 'Mn' model. Even if the upward concave-convex (or sigmoid) relation could easily be subdivided into three segments roughly corresponding to a primary, a secondary and a tertiary range, these ranges were continuously connected and no jump or firing acceleration as that in the 'Mn' model were present (Paper II). The transition between the various firing ranges was also taking place without any special trajectory event, i.e. there was no special primary, secondary or tertiary range trajectory behaviour (compare Fig. 3 A and filled circles Fig. 3 C).

In the trajectories computed without a DD (see Fig. 3 B) the scoop was given by the brief hyperpolarizing overshoot directly followed by a ramp displaying a considerable upward convex curvature. With increasing current, this ramp given by the exponential $G_K(t)$ decay rotated (in

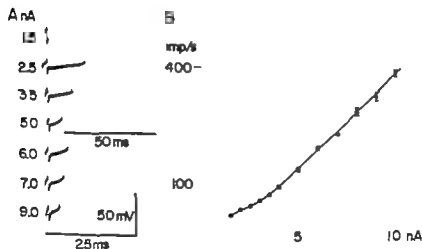


Fig. 4 Trajectory shape and first interval f/I relation for a DSCT neurone. In the f/I relation in B is given for each current strength the average value (filled circles) \pm S.D. ($n = 4-6$). (At some current strengths the \pm S.D. is within the area of the filled circles). Observe that the transition between the lower segment of the f/I relation and the upper, steeper segment occurs without any change in trajectory shape. Compare the trajectories in A with those in Fig. 3 A.

much the same way as the scoop given by the $G_K(t)$ plateau in the "Mn" model) around the pivot point given by the falling phase of the spike. The corresponding f/i relation (Fig. 3 C open circles) did, however, display the same sigmoid shape as with a DD (filled circles) but in a much more compressed form. Also with this model version there was no direct correlation between trajectory shape and f/i shape (compare Fig. 3 B and 3 C open circles).

In previous investigations (Kuno and Miyahara 1968) the first interspike interval f/i relation in DSCT neurones was only studied in the higher frequency range, where an upward convex relation, as that demonstrated by the model was found. The trajectory records given displayed the same upward convex-concave-linear shape as given by the DD-scoop-ramp sequence in the computations in Fig. 3 A. In the present study the first interval f/i relation was examined in 11 DSCT neurones over a much larger frequency range especially at the lower frequencies and the same sigmoid shape as found in Fig. 3, was present in all these neurones (Paper V). Due to the invariable presence of a DD both the f/i relations and the trajectories corresponded better to the model version in A than in B. The first interval trajectories and the corresponding f/i relation from a typical DSCT neurone is shown in Fig. 4 (tertiary range not illustrated). Observe in Fig. 4 A that the trajectory modifications with increasing current are very similar in this neurone to that of the model in Fig. 3. As in the model no correlation between the trajectory behaviour and f/i shape was found. In none of these 11 cells was there any sudden jump or discontinuity in the f/i relation or a sudden change in trajectory shape. The small $G_K(t)$ plateau probably present in some DSCT neurones gave in these cells a slight upward convex trajectory shape, preceding the subsequent ramp decay.

A firing behaviour similar to that described in Fig. 3 B and C (open circles) was not often found in DSCT neurones (see however Fig. 9). However, in other central neurones such as the rubrospinal neurones (Ruitborn, Murakami and Tsukahara, to be published) and cells belonging to the ventral spinocerebellar tract (VSCT) such behaviour is more easily found, as illustrated from a VSCT neurone in Fig. 11. Note in Fig. 5 A the marked upward convex ramp and its rotation with increasing current. In the corresponding f/i relation in Fig. 5 B the upward concavity is hardly present and is followed at rather low frequencies by an upward convex shape as in the model version in Fig. 3 B.

The first interval f/i relation in DSCT neurones is then descriptively very similar to that found in motoneurones with a 'primary', 'secondary' and 'tertiary' range of firing. Despite this similarity the underlying mechanisms are not quite the same, as also unveiled by the different trajectory behaviour between motoneurones and DSCT neurones with



Fig 5 Trajectory shape and first interval f/I relation for a VSCT neurone. In the f/I relation in B is given for each current strength the average value ($n = 4-6$). Compare the upward convex ramp in A with that in Fig 3 B and the corresponding f/I relation in B with that in Fig 3 C (open circles). (Gustafsson, unpublished)

the absence of the scoop inversion (secondary range trajectories) in the latter cells. The particular role of the DD in the "DSCT" model for the high frequency firing and for the shape of the subsequent trajectories (compare the ramp shape in Fig 3 A and B) should be recognised.

B Computed adaptation in the neurone model

In central neurones studied (Kernell 1965 b; Eide et al. 1969; Kolke et al. 1970; Baker and Precht 1972) there is a rapid decrease in discharge frequency after the application of a constant current pulse (adaptation). The AHP summation has been considered to be of major importance for this adaptation (Kernell 1963, 1972; Calvin and Schwindt 1972). In α -motoneurones and DSCT neurones the adaptation has been shown to be most pronounced at higher currents and to be largely over within the first few interspike intervals (Kernell 1965 b; Eide et al. 1969). From the experimental evidence given in the preceding section, the AHP summation in the neurone model was as a first approximation considered to be algebraical. To account for the variability from algebraical summation found in real neurones e.g. the AHP depression a correction factor was also introduced. In this section the results from computations based on this model will be taken up. Since the adaptation is largely over within the first interspike intervals, the computations were only performed for the first three intervals following the onset of a current pulse.

much the same way as the scoop given by the $G_K(t)$ plateau in the "Mn" model) around the pivot point given by the falling phase of the spike. The corresponding f/I relation (Fig. 3 C open circles) did, however, display the same sigmoid shape as with a DD (filled circles) but in a much more compressed form. Also with this model version there was no direct correlation between trajectory shape and f/I shape (compare Fig. 3 B and 3 C open circles).

In previous investigations (Kuno and Miyahara 1968) the first interspike interval f/I relation in DSCT neurones was only studied in the higher frequency range, where an upward convex relation, as that demonstrated by the model, was found. The trajectory records given displayed the same upward convex-concave-linear shape as given by the DD-scoop-ramp sequence in the computations in Fig. 3 A. In the present study the first interval f/I relation was examined in 11 DSCT neurones over a much larger frequency range, especially at the lower frequencies and the same sigmoid shape as found in Fig. 3 was present in all these neurones (Paper V). Due to the invariable presence of a DD both the f/I relations and the trajectories corresponded better to the model version in A than in B. The first interval trajectories and the corresponding f/I relation from a typical DSCT neurone is shown in Fig. 4 (tertiary range not illustrated). Observe in Fig. 4 A that the trajectory modifications with increasing current are very similar in this neurone to that of the model in Fig. 3. As in the model, no correlation between the trajectory behaviour and f/I shape was found. In none of these 11 cells was there any sudden jump or discontinuity in the f/I relation or a sudden change in trajectory shape. The small $G_K(t)$ plateau probably present in some DSCT neurones gave in these cells a slight upward convex trajectory shape, preceding the subsequent ramp decay.

A firing behaviour similar to that described in Fig. 3 B and C (open circles) was not often found in DSCT neurones (see however Fig. 9). However, in other central neurones such as the rubrospinal neurones (Hultborn, Murakami and Tsukahara, to be published) and cells belonging to the ventral spinocerebellar tract (VSCT) such behaviour is more easily found, as illustrated from a VSCT neurone in Fig. 5. Note in Fig. 5 A the marked upward convex ramp and its rotation with increasing current. In the corresponding f/I relation in Fig. 5 B the upward concavity is hardly present and is followed at rather low frequencies by an upward convex shape as in the model version in Fig. 3 B.

The first interval f/I relation in DSCT neurones is then descriptively very similar to that found in motoneurones with a 'primary', 'secondary' and 'tertiary' range of firing. Despite this similarity the underlying mechanisms are not quite the same, as also unveiled by the different trajectory behaviour between motoneurones and DSCT neurones with

for the later intervals. No crossing between the second and third interval f/I relations was present, demonstrating the role of the $G_K(I)$ plateau for that behaviour. (Such a crossing could, however, also be present if the first interval had "jumped" to the peak of the DD). In the computed trajectories the DD reached a less depolarized voltage and the scoop was more pronounced for the later intervals.

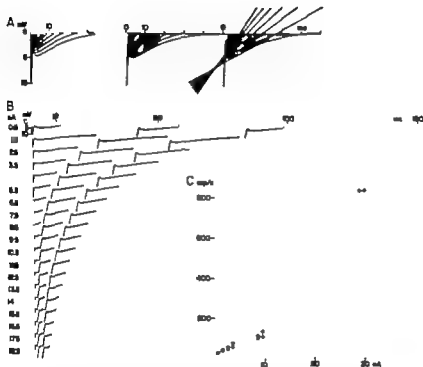


Fig. 6 Trajectory shape and f/I relations for the first three intervals in the "DSCT" model with algebraical summation. A First, second and third interval trajectories are shown superimposed for each interval on a common starting point. The straight lines are fitted to the ramp slopes by eye. B Same trajectories as in A but displayed sequentially. C Relation between the current injected and the reciprocal of the first (triangles), second (open circles) and third (filled circles) intervals. For details see text.

3 Computation without conductance plateau and with AHP depression Due to the presence of an AHP depression in DSCT neurones the same computation was performed after multiplication of the second and third interval AHP $G_K(t)$ with a factor of 0.5 (Fig. 7). The second and third interval f/I relations then derived displayed the same general course as for algebraical summation with linearization and increase in primary-to-secondary range transition level, but were displaced to the left in the graph crossing the first interval f/I relation twice. At low frequencies (< 100 imp/s) the second and third interval f/I relations were to the left of the first interval f/I relation, i.e. there was an initial firing acceleration. With higher current the first interval f/I relation was crossing the other two relations i.e. indicating an initial adaptation. However with still higher current, first the second, then the third interval were again crossing the first interval, i.e. representing again an initial firing acceleration. In the corresponding trajectory records the peak of the DD reached a more depolarized level, the scoop was less pronounced and the ramp less steep for the later intervals than for the first interval (see Fig. 7 A). i.e. the contrary from the trajectory behaviour with algebraical summation.

Among the 11 DSCT neurones analysed for the three first intervals firing behaviour both patterns described above were found (Fig. 8 and 9). Note, however that the second and third interval f/I relations are somewhat more displaced to the right in respect to the first interval f/I relation in the experimental relations than in the model, i.e. the adaptation is somewhat more pronounced or acceleration less pronounced in the real neurones than in the model. This phenomenon is most likely caused, as was also described for motoneurones (Paper II) by the fact that the voltage displacement given by a constant current pulse does not remain constant but decays in time due to a still unknown process (Ito and Osima 1965; Nelson and Lux 1970) giving a difference in threshold current between the first few spikes. In three other neurones with marked AHP depression the firing acceleration was only found at the high frequency region and not at the lower frequencies (< 100 imp/s). The f/I slopes in the low frequency region for the second and third intervals were, however in these neurones steeper than for the first interval demonstrating that the adaptation in these neurones was only caused by the current threshold difference described above shifting the f/I curves to the right. In the remaining six neurones analysed, no firing acceleration was found at any frequency level and the second and third interval f/I slopes were also less steep than for the first interval. Only in one of the cells analysed was there a tendency for a second third interval crossing as that demonstrated for motoneurones. A crossing between the first and the second-third interval f/I relations as described above, was not found in the motoneurones analysed.

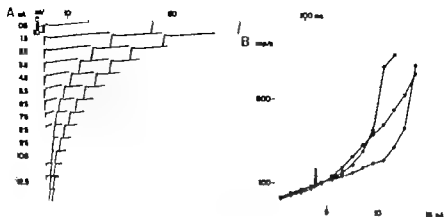


Fig 7 Trajectory shape and f/I relations for the first three intervals in the "DSCT" model with AHP depression A Trajectories for the three intervals displayed sequentially B Relation between current injected and the reciprocal of the first (triangles) second (open circles) and third (filled circles) intervals For details see text

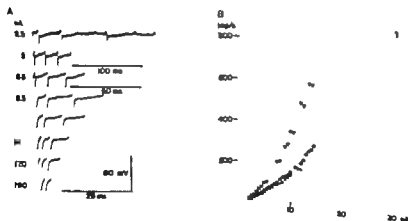


Fig 8 Trajectory shape and f/I relations for the first three intervals in a DSCT neuron In the plot is given the average value of 4-6 trials at each current strength (500 ms pulses at a frequency of 0.2/s) Same symbols as in Fig 7 and 5 For details see text

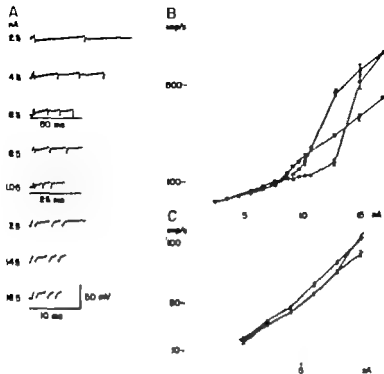


Fig 9 Trajectory shape and f/I relations for the first three intervals in a DSCT neurone. In the plot is given the average value \pm S.E. of 4-6 trials at each current strength (500 ms pulses at a frequency of 0.2/s). Same symbols as in Fig 8. For details see text. In C is shown an enlargement of the lower part of the graph in B.

Thus the predictions of the model do not only account for the general features of the initial adaptation in DSCT neurones, such as the increase in linearisation of primary range and the transition level to secondary range with successive intervals, but also more specifically for the presence of the two phases of firing acceleration given by the AHP depression. The difference in the firing behaviour between motoneurones and DSCT neurones already found for the first interval is also more accentuated (lack of second-third interval crossing in DSCT neurones and lack of first-second interval crossing in motoneurones) and is apparently accounted for by the difference in AHP properties.

As demonstrated in Paper III a model based on an exponential $G_K(t)$ decay and an algebraical summation will for the steady state firing, i.e. when the adaptation is completed display a slightly upward convex, almost linear relation up to very high frequencies. Such a model will thus not display a 'secondary' range. On the other hand, in the presence of a pronounced $G_K(t)$ plateau the steady state f/I relation will bend upwards at higher frequencies thus displaying a secondary range of firing. A model based on the AHP $G_K(t)$ time course and algebraical summation is then able to explain the original difference described between motoneuronal firing behaviour and that of the DSCT neurones namely the presence of a 'secondary' range in some of the former cells and its absence in the latter cells (Kernell 1965 c; Elde et al. 1969).

In the steady state firing in motoneurones with a pronounced $G_K(t)$ plateau the 'secondary' range should be reached when the frequency exceeds that corresponding to the end of the plateau phase (or approximately the 1/time-to-peak AHP). It is however reported that the transition frequency also for the steady state firing can be appreciably lower than this limiting frequency (Kernell 1965 d). Moreover the ratio between the steady state 'secondary'/'primary' f/I slopes can be appreciably higher in real neurones (Kernell 1965 c) than that given by the model, thus implicating an additional mechanism for the 'secondary' range of firing in motoneurones. It should, however be recalled that the algebraical summation is only a first approximation to the AHP summation mechanism in motoneurones and, in fact, in many of the motoneurones analysed the summation was appreciably less than algebraical at shorter intervals. In fact, if the summation is less than algebraical at shorter intervals and algebraical at longer intervals, i.e. if the correction factor is not constant but decreases with decreasing interval, then the 'secondary' range would be expected to start at lower frequencies and deviate more from the 'primary' range f/I slope than that predicted by algebraical summation. Motoneurones and DSCT neurones may then not only differ with respect to the $G_K(t)$ time course but to some extent also to the AHP summation. However in the present investigations the f/I slope of two DSCT neurones was also somewhat steeper at higher frequencies than at lower. In motoneurones with less developed $G_K(t)$ plateau the firing would be more similar to that of the DSCT neurones i.e. the steady state 'secondary' range could even be (if algebraical summation) hardly present in such cells. This prediction then fits well with the recent observation (Schwindt 1973) that motoneurones lacking steady state 'secondary' range even up to very high discharge frequencies have a shorter AHP duration and thus likely a shorter time-to-peak AHP (Kernell 1965 d) and display more adaptation than neurones giving steady state 'secondary' range.

Even if the firing behaviour of a model based on the AHP conductance time courses (and the DD current) is fairly similar to that found in real neurones there are several aspects of the firing in real neurones not accounted for by such a model

The adaptation in the model is given by the AHP summation and is completed within the first few interspike intervals. The presence of adaptation in cells displaying an AHP depression and the appreciably longer adaptation found in both motoneurones (Granit et al. 1963 b; Kernell 1965 b) and DSCT neurones (Eide et al. 1969) demonstrates that other mechanisms must be included. As discussed previously the 'sag' in the voltage given by a constant current pulse is likely to give rise to the current threshold difference between the first few spikes and thus to the adaptation found in cells with AHP depression. A longlasting adaptation has also been found in invertebrate neurones (Sokolove and Cooke 1970) and has there been attributed to the activation of an electrogenic sodium pump. The existence of such a sodium pump has also been verified in vertebrate neurones by the injection of Na^+ ions through the recording microelectrode (Ito and Oshima 1965 a, b; Kofke, Mamo, Okada and Oshima 1972). In pyramidal tract (PT) neurones a slower hyperpolarizing component of the AHP appearing after a long (> 20 spikes) train of spikes at high frequency has been attributed to the activation of this sodium pump and has been considered as a mechanism for a long-lasting adaptation.

In the present investigations the afterpotentials following long spike trains of high frequency were studied in 12 DSCT neurones and a typical finding is exemplified in Fig. 10 from two neurones. The posttetanic hyperpolarization (PTH) so evoked, was hardly noticeable with spike trains below a few seconds (100 Hz frequency) and increased thereafter in proportion to the train duration. The peak voltage of the PTH was usually not reached until a couple of seconds after the end of the train and decayed thereafter slowly during 80-100 s. There was no apparent conductance increase during the PTH, as judged from the voltage drop caused by current pulses (see Fig. 10 B) indicating that it is caused by an active process such as a sodium electrogenic pump. This PTH needs then more spikes and lasts considerably longer than that found in PT neurones. Its time course is however fully compatible with that expected from an electrogenic pump (Ito and Oshima 1965 a, b; Kofke et al. 1972). The slow onset and the number of spikes needed to evoke this PTH would indicate that it hardly can account for much of the adaptation in motoneurones and DSCT neurones during the first few seconds of a discharge, but rather could be responsible for the later adaptation in

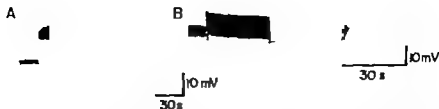


Fig 10 Post-tetanic hyperpolarization (PTH) in DSCT neurones In A and B are shown the hyperpolarization following a longlasting tetanic (100/s) antidromic (cerebellum) activation in two DSCT neurones. Observe in A the increase in PTH with increased number of spikes. In B can be observed the constancy in the voltage deflection given by a train of constant current pulses throughout the PTH. For further details see text.

motoneurones described by Gravit et al (1963 b) and also found in DSCT neurones (Gustafsson, Lindström, Takata and Zangger to be published).

In the model there is no frequency limiting process implying that the steady state frequency can reach the highest values. While the steady state firing in DSCT neurones can reach very high frequencies (up to 600 imp/s; Elde et al. 1969) the steady state firing in motoneurones in response to constant current injection never exceeds 150-200 imp/s (Kernell 1965 c) and very often the steady firing fails at considerably lower frequencies. Whether this difference is caused by the larger amount of current needed to reach high frequencies in motoneurones than in DSCT neurones, by a higher sensitivity in motoneurones for inactivation given by insufficient repolarization between the spikes or by the presence of the brief overshawing in DSCT neurones preventing inactivation, is at present not known. It should be noted that the DSCT neurones invariably despite the apparent injury of these cells upon penetration (Elde et al. 1969) responds to constant current injection with a continuous discharge (see also Elde et al. 1969) and do not show phasic behaviour as some motoneurones (Mishelovich 1969) indicating some fundamental difference between DSCT neurones and at least some motoneurones. To which extent the inactivation contributes to the adaptation in continuously firing neurones is unclear.

In the model only the K conductance is allowed to summate or change with repetitive activation. In motoneurones (Kernell 1964) and in DSCT neurones (Paper IV) the DD can also increase with successive spikes a feature which in one DSCT neurone investigated was of decisive importance for its firing behaviour. In this neurone the AHP following a single spike was hardly noticeable, increasing slowly with successive spikes. This allowed the DD to overcome the AHP for the first few spikes and the neurone was subsequently responding with a short burst of spikes terminated by the AHP summation, the bursts increasing in frequency with increasing current. The DD increase served thus as an intrinsic mechanism for burst firing emerging in the presence of a minute AHP. This multiple-spike response of the DSCT neurones was somewhat similar to that found in the pyramidal cells in hippocampus (Kandel and Spencer 1961) or in neurones in the inferior olive nucleus (Crill 1970; see also Calvin 1974). In the pyramidal cells the burst was however terminated by a sodium conductance inactivation and not by the AHP summation.

A AHP and firing control

It is generally agreed that for the understanding of the properties of the rhythmic firing in neurones with their low frequency firing ability and their large frequency range, long time constant processes not included in the Hodgkin-Huxley equations for the squid axon (Hodgkin and Huxley 1952) must be visualized. Since little is known about such long time constant processes models trying to simulate the firing behaviour of central neurones have either been formal, including a long decay of a simple threshold function (Kernell 1968 Pertile and Harth 1971) or been modifications of voltage clamp equations from squid axon (Shapiro and Lenherr 1972) or myelinated nerve fibre (Kernell and Sjöholm 1972, 1973). However as stated in the Introduction, even such an elaborated model as that of Kernell (1972, 1973) is still not accounting for the specific firing behaviour of central neurones. It may be asked if this difference is caused by the specific properties of the processes underlying the afterpotentials in neurones not accounted for by modified voltage clamp equations from axons or by the influence of other factors such as the spatial distribution, not easily foreseen from the axonal properties.

In the Introduction two alternative hypotheses for explaining the firing behaviour of central neurones were brought up. On one hand, the firing behaviour was determined by the time course of the refractoriness, on the other the changes in spatial distribution of the AHP was considered to be critical. The results presented now are clearly favouring the former view. The trajectory modifications and f/I relations given by the model based on fixed postsynaptic conductance processes are very similar to those found in the real neurones. Thus neither the trajectory behaviour described by Schwindt and Calvin (1972) and Schwindt (1973) nor the sudden change in trajectory shape when entering the 'secondary' range, described by Kernell (1965 c) are, in fact, indicating that any change in spatial distribution of the AHP given by increasing current injection should occur. The difference between the f/I relations and trajectory behaviour in motoneurones and DBCT neurones seems also to be a consequence of a difference in the respective AHP conductance properties. The effect of synaptic currents during rhythmic firing has also recently been re-examined (Schwindt and Calvin 1973 a) and no effects such as those reported by Granit et al (1966 b) could be found. It seems therefore that very little evidence exists that changes in the spatial distribution of the AHP should play any decisive role in firing regulation.

This conclusion does however not mean that the spatial distribution of the antidromic invasion could not be of importance for the firing behaviour. An alternative view on the effect of a spatial factor was recently forwarded in a preliminary note (Mauritz, Schlue, Richter and Nacimiento 1973). In this paper it was suggested that the plateau in the conductance time course could be explained by a limited dendritic invasion during this phase, thus indirectly re-emphasising the role of the neuronal geometry. For example, the lack of 'secondary range' in DSCT neurones could then be a consequence of a higher safety factor for impulse transmission in these cells as suggested by Eide *et al* (1969). The hypothesis that the plateau is given by a limited dendritic invasion is neither supported nor clearly invalidated by the present findings. It was, however stated in that note that "the limited dendritic invasion increases with each additional spike until a maximum is reached for the corresponding current strength". This postulated increase in dendritic invasion with the later intervals removing the plateau, seems somewhat in conflict with the successive changes in the first, second and third interval trajectories during adaptation displayed by real neurones and well fitting with the algebraical summation. Moreover the lack of effects of synaptic currents on the f/I relations reported by Schwindt and Calvin (1973 a) and the relative stability of the plateau phase with subthreshold polarizations and with suprathreshold currents seems also to indicate that the plateau is more fixed than expected if governed by a spatial factor (see also below).

A model for repetitive firing regulation in motoneurones based on fixed only time-dependent postspike conductance changes has recently been criticized from another angle than from the consideration of spatial factors (Schwindt and Calvin 1973 b). In that paper it was suggested that the firing in motoneurones is regulated by a time and voltage dependent potassium conductance and a possible subthreshold sodium conductance change which is time and voltage dependent. This latter conductance should be responsible for the development of the 'secondary range' trajectory shape and subsequently to the 'secondary' range of firing.

From scrutiny of the experimental results reported by Schwindt and Calvin (1973 b), it is clear that only those results concerning the conductance measurements are opposed to that which would be predicted by the model. In fact, due to the presence of the AHP summation no linear relation between scoop depth and current injected would be found in the model, a behaviour thus confirmed in real neurones by Schwindt and Calvin (1973 b). Also the findings presented in the *downstep* experiments are clearly in line with the model behaviour.

The model would, however not predict the linear relation between the scoop depth and the inverse of current injected reported. This is no fault of the model.

since such a linear relation should only be present in the motoneurons in Fig. 4 III (Schwindt and Calvin 1973 b) if the threshold current (which should be of no interest in this respect) is included.

On the other hand, the model would not predict the findings that the interspike interval (ISI) conductance should be lower (or the same) during a short ISI than during a long ISI or at resting level, and that it should be lower than the resting conductance in the later part of the ISI. According to Schwindt and Calvin these results should indicate the presence during the ISI of a voltage dependent sodium conductance evoked by the spike (see also Discussion, Paper I).

There are, however, some evidence that the conductance time courses given by Schwindt and Calvin should not be taken for granted. In fact, other authors (Mauritz et al. 1973) have recently reported measurements of conductance time courses during rhythmic firing and found similar time courses as that during resting conditions i.e. with conductance increase and presence of a conductance plateau. Moreover in the experiments by Schwindt and Calvin it was not always possible to maintain the bridge balance, which could easily give erroneous results since an incompensation should not only affect the absolute conductance but also its time course. It should also be noted that the findings of constant conductance in the early part of the ISI with increasing currents seems to be in contradiction to the above-mentioned finding of a nonlinear relation between scoop depth and current injected. There are, however, no reasons to doubt that the huge increases in pulse height occurring at the very end of the ISI to some extent can be real and be produced by the activation of a sodium conductance giving the succeeding spike. Such a sodium activation is, in fact, also indicated by the upward deviation of the experimental trajectories at the very end of the ramp (see, for example, Fig. 2 Schwindt and Calvin 1972) not present in the computed trajectories. The importance of that conductance for firing regulation would however be far from that suggested for the hypothetical sodium conductance of Schwindt and Calvin.

In the present proposed model the K conductance underlying the AHP is treated as completely separated from the K conductance underlying the falling phase of the spike. This separation was suggested for motoneurons already by Eccles (1957) and is also a feature of the Kernell model (Kernell and Sjöholm 1972). The AHP K conductance was considered by Eccles to increase slowly after the spike, reaching a late peak, corresponding to the peak of the AHP voltage, thereafter slowly decaying. In the Kernell model the AHP K conductance displays a simple exponential decay starting during the descending phase of the spike. From the finding of a selective depression of the AHP evidence is now at hand that the AHP K conductance in fact is separated from the K conductance underlying the falling phase of the spike. From the conductance computation and measurements in motoneurons it would be tempting to accept the suggestion of Eccles. However, from the time courses of the depressed AHPs in DSCT neurones it seems, however, as if the whole computed time course, including also the initial fast $G_K(t)$ decay, is belonging to the AHP K conductance.

As mentioned above, it was recently suggested that a limited dendritic invasion could account for the plateau, thus giving an explanation for the odd and (especially in motoneurons) perhaps even unlikely (in respect to Hodgkin-Huxley equations) AHP $G_K(t)$ configuration. The coupling existing between the time-to-peak AHP (or plateau duration) and the duration of the AHP (or decay time constant) even if somewhat variable (Kernell 1965 d) seems however to suggest that the plateau is given by the time course of the ionic kinetics rather than by a spatial mechanism (see also above). The existence of K conductance systems not included in the Hodgkin-Huxley equations has also been recently described in molluscan neurones (Conner and Stevens 1971, Gola 1974) indicating that other ionic mechanisms than those described for axons can operate in the soma-dendritic membrane of central neurones.

The concept of algebraical summation, i.e. that the AHP conductance is not affected by the following spikes and that each spike is evoking the same K conductance was introduced as a first approximation for description of the AHP summation. Even if it is clear from the experimental analysis that each spike is not evoking the same K conductance the idea that the AHP conductance is not affected by the succeeding spikes has been successful for explaining the successive trajectory changes during adaptation in motoneurons and of the summation-depression pattern and initial high frequency firing acceleration in DSCT neurones. In the Kernell model an AHP summation can be present if the rate constant for the AHP K conductance is chosen so that the peak value of that conductance is not reached during the first spike. Due to the con-

ditioning effect of the remaining conductance, a higher one will be evoked by the next spike. This model will thus explain neither the long-lasting depression nor the difference in $G_K(t)$ time course between the AHPs following one and two spikes.

In the model there will be an equivalence between current injected through a microelectrode and currents given by synaptic conductance changes. Even if such an equivalence exists in many motoneurons under synaptic drive from different sources (Schwindt and Calvin 1973 a, Granit et al. 1966 a, b) a change in the f/I slope in both the 'primary' and the 'secondary' range of firing has been reported (Kernell 1965 a, Granit, Kernell and Lamarre 1966 b, Shapovalov, Kurchavii and Stroganova 1966, Shapovalov 1972). If this behaviour is caused by the interaction of synaptic and AHP conductance changes in the dendritic tree (Kernell 1971) or by other factors (Granit et al. 1966 b) is at present unknown.

C Descriptive terminology

Many previous studies of the rhythmic firing in motoneurons have mainly been concerned with describing the f/I relations and trajectory behaviour. The f/I relations and trajectories have been linearized and related to each other via simple geometrical schemes (Schwindt 1973). Even if it was appreciated that these descriptions mainly served as constraints for future modelling, conclusions regarding possible underlying mechanisms based on these simplistic schemes were drawn (Schwindt and Calvin 1972, Schwindt 1973). The different trajectory behaviour found between motoneurons displaying e.g. "stereotyped" ramps and some other CNS neurons displaying e.g. ramp slope changes were then taken as indications of different firing mechanisms (Schwindt and Calvin 1972, Schwindt 1973).

The present studies have instead started the analysis from a different angle, deriving the f/I relations and trajectories from a postulated underlying mechanism, covering not only motoneurons but also with minor modifications D&CT neurons. The firing then derived displayed characteristics well within the general framework given by previous investigations. There were, however, many deviations in the model behaviour from the simple schemes earlier suggested. The f/I relations were not linear and thus not easily dividable in different firing ranges and the trajectory changes were not as clear cut as described. No easy correlation could be made between various segments of the f/I relations and of the trajectories, especially not when comparing the various model versions. It is apparent from the presented results that the same more complex firing behaviour in fact is present also in the real neurons.

and that thus the same difficulty in imposing simple firing range subdivision and trajectory description is present also for them. Most of the nomenclature earlier given is thus not helpful when interpreting the underlying mechanism but is rather obscuring it. In fact, different f/I shapes can be given by the same underlying mechanism and different underlying mechanisms can give the same f/I shape. Different trajectory shapes can be given by the same underlying mechanism and the same trajectory shape can be given by different underlying mechanisms. These statements are exemplified by considering that the f/I relation and the ramp behaviour given by an exponential decay is much dependent upon the initial phase of the AHP and on the DD. The upward bending of the f/I relation or a 'secondary' range can result from an exponential decay and a plateau. The upward convex trajectory shape can originate from a DD, a plateau and even an exponential $G_K(t)$ decay.

Even so it is obvious that a correlation between the underlying mechanisms, the f/I shape and the trajectory shape exists and thus that a scrutiny of e.g. the trajectories will disclose both the expected f/I relations and the underlying mechanism. The present results do however demonstrate that in such a correlation the whole sequence of trajectories must be considered as a unity and the interpretation of a trajectory must be related not only to its position in the interspike interval and when it appears with increasing current but also in relation to the trajectory shapes preceding it. By such an analysis the f/I relations could be predicted, as well as the underlying mechanism. Such predictions given by the model analysis and verified in real neurones are easily exemplified. For example, the presence of a small DD, an early deep scoop and an upward convex ramp is indicating from the model an almost linear to upward convex f/I relation already for the first interval, a finding also verified in real neurones. An expressed DD and a shallow early scoop followed by an almost linear ramp (changing in slope with increasing current) gives the sigmoid first interval f/I relation and a slightly upward convex, almost linear steady state f/I relation. The presence of an upward concave scoop gradually shifting to upward convexity is indicative of a conductance plateau, possibly giving firing jumps for the first interspike intervals and an upward deviation of the f/I relation at frequencies exceeding $1/\text{time-to-peak AHP}$.

D Other CNS neurones

In a few other CNS neurones some data are available on the AHP and the firing properties. These neurones can, interestingly enough, be subdivided into two groups grossly comparable to α -motoneurones and D&CT neurones respectively. On one hand, the trochlear (TMN;

Baker and Precht 1972) and Paolal (FMn) motoneurons (Kitai et al 1973) and the pyramidal tract (PT) cells (Takahashi 1965) display AHPs with rather long time-to-peak (8-10 ms in TMn and FMn and appreciably longer in PT cells) showing summation and no depression with repetitive activation. A steady state secondary range is also present in PT neurones (Kofke et al. 1970) and an upward concave f/I relation can also be found in TMn (Baker and Precht 1972; FMn not investigated in this respect). No increase in firing rate with time has been found in TMn and slow PT cells. In fast PT cells, however, a firing acceleration comparable to that in DSCT neurones at a short first interspike interval has been found (Kofke et al 1970). On the other hand the rubro- (Tsukahara, Toyama and Kosaka 1967) and vestibulospinal (Ito Hongo Yoshida, Okada and Obata 1964) neurones exhibit an AHP with a very short time-to-peak (1-3 ms) i.e. even shorter than DSCT showing no summation (Tsukahara et al 1967 Ito et al. 1964) or in the rubrospinal neurones even an AHP depression (Hultborn, Murakami and Tsukahara, to be published) i.e. a behaviour very similar to that in DSCT neurones. In the rubrospinal neurones the first interspike interval firing is very similar to that shown in Fig. 3 and in the steady state secondary range is usually not found (Hultborn, Murakami and Tsukahara, to be published).

E Functional implications

In the preceding section it was suggested that neurones discharging repetitively in a steady fashion could be subdivided into at least two groups with respect to the AHP properties and the firing behaviour. A coupling between the firing behaviour displayed by neurones belonging to these two groups and their function has been made by previous authors (Kernell 1965 d, 1966 Elde et al 1969). For the α -motoneurones it was suggested (Kernell 1965 d) that the presence of a secondary range in the steady state firing could be present as a compensation for the decline in the muscle tension-firing frequency relation at higher frequencies. This coupling between firing behaviour and muscle properties is then well in line with the fact that the other neurones grouped together with the α -motoneurones except the PT cells are directly connected to muscles. It has however been questioned to which extent the motoneurones in steady firing are reaching these high frequencies and thus the usefulness of this mechanism (Schwindt 1973 see, however Kernell 1965). On the other hand, high discharge frequencies are more easily evoked initially in the discharge, a behaviour which was then considered (Kernell 1966) in relation to the fact that a muscle requires much higher stimulus rates for a maximal rate of rise of tension than for the maintenance of a maximal tetanic contraction (Buller and Lewis

1965) That the initial firing behaviour could be of importance in relation to the muscle properties is more emphasized by the catch property (Wilson and Larimmer 1968) also found in mammalian muscles (Burke, Radomin and Zajac III 1970) i.e. that the tension given by a low frequency firing is greatly enhanced during a considerable time by an initial short interspike interval. The AHP mechanism in motoneurons with a $G_K(t)$ plateau, giving rapid shortening of the first interspike interval, and an AHP summation giving a low succeeding frequency is then well suited for use of this muscle property. The steady state secondary range might then rather in that context, be considered as a *spin off* behaviour. An initial short interspike interval followed by a low frequency discharge is in fact, also the manner in which fast movements are initiated in human muscles (Gurfinkel, Mirskii Tarko and Surguladze 1972). As in the motoneurone model and as in the real neurones this short first interval is characteristically also followed by a longer second than third interval. By comparing the records in Fig 1 (Gurfinkel et al 1972) where the first, second and third intervals are 72 12 and 18 imp/s respectively and e.g. the graph in Fig 7 B Paper III, where the corresponding intervals at 20 nA are 64 13.5 and 15.5 imp/s it is clear that the AHP is quantitatively capable of producing the firing behaviour described by Gurfinkel et al. without any contribution from other mechanisms.

The linear steady state f/I relation found in DSCT neurones is easily imagined as appropriate for their function, since with such a relation a given synaptic input will give the same frequency increase regardless of other synaptic activity (Eide et al 1969). Since these neurones are most likely firing more or less continuously i.e. being steadily adapted, the upward deviations or secondary ranges found for the first interspike intervals with constant current injections might then rather be a *spin off* behaviour just indicating the ingenious manner in which a linear steady state f/I relation is achieved through a linear summation of exponential decays. A similar reasoning might also be applicable to the rubro- and vestibulospinal neurones also not directly coupled to the muscles. The significance of the AHP depression is not easily understood. It is however clear that the depression is a very effective means of converting the f/I shape from the sigmoid shape of the first interval to the 'desired' steady state linearity without much adaptation, which might be an unnecessary and even unwanted feature of neurones not coupled to muscles.

It was recently suggested that the trajectory behaviour in motoneurons during primary range firing was well suited for maintaining a constant firing behaviour in the presence of accommodation and synaptic activity (Schwindt 1973). This suggestion was based on the implicit assumptions that the ramp in motoneurons was stereotyped

and that the input resistance was constant throughout the ramp. Since neither the ramp is stereotyped nor the resistance constant this suggestion seems hardly warranted.

In the present studies the AHP properties in both α -motoneurones and the dorsal spinocerebellar tract (DSCT) neurones were investigated in the cat. A neurone model based on the AHP mechanisms and on some simplifying assumptions of the neurone properties was then derived. The firing behaviour of this model was computed and a comparison between the model firing and the firing behaviour of the real neurones was performed.

From these investigations the following conclusions were drawn

1 The AHP in DSCT neurones is given by a K conductance process as earlier described for motoneurones (Coombs et al 1955) and not by a sodium electrogenic pump (Kuno et al 1970) or by a sodium conductance decrease (Elde et al 1969)

2 The K conductance underlying the AHP is separated from that giving rise to the falling phase of the spike as suggested previously (Eccles 1957). In motoneurones this K conductance describes a complex time course with an initial fast decay, a plateau phase and a subsequent slow exponential decay. In DSCT neurones the peak AHP voltage is substantially smaller and is reached earlier than in motoneurones. This is correlated with a lack or poor development of the plateau phase in the conductance time course in DSCT neurones. The AHP conductance was found to be only little affected by subthreshold potential variations. Equations describing the conductance time course in both motoneurones and DSCT neurones were given.

3 In both motoneurones and DSCT neurones the AHP summation can, as a first approximation, be described as an algebraical process. The absolute summation can, however, be larger or smaller than the algebraical summation. Some DSCT neurones, besides showing AHP summation, can also present a temporal depression of AHP, i.e. the AHP following two spikes is less than that following a single spike.

4 The frequency-current (f/I) relation in the model based on the AHP conductance time course in motoneurones displays the three firing ranges found in real motoneurones (Kernell 1965 & Schwindt 1973). With increasing current injection the interspike voltage trajectories are also undergoing the same peculiar modifications as described for real motoneurones (Schwindt and Calvin 1972; Schwindt 1973). A re-examination of the first interspike interval firing in real motoneurones revealed features predicted by the model, not observed before. It is concluded that the model is well simulating the firing behaviour of the first interspike interval in motoneurones.

5 Implemented with an algebraical summation of the AHP conductance following successive spikes the same model simulates well the successive changes in the f/I relations and the interspike voltage trajectories found in real motoneurons for the initial intervals after the onset of a constant current. The steady state f/I relation of the model can also display an upward deviation from linearity at higher current intensities i.e. a secondary range of firing as described for real motoneurons (Kernell 1965 c). It is concluded that the AHP conductance is the decisive factor for determining the f/I relation as well as the interspike voltage trajectories in motoneurons.

6 The firing behaviour of a neurone model based on the AHP conductance time course in DSCT neurones and on a shortlasting depolarising current giving rise to the delayed depolarisation simulates adequately the first interspike interval f/I relation and trajectory behaviour in these cells as experimentally determined in the present investigations. Even if the f/I relation in DSCT neurones (and the model) could be descriptively subdivided into three firing ranges as in motoneurons the underlying mechanism for this behaviour is *not* quite the same as for motoneurons as also unveiled by the *different* trajectory behaviour.

7 The firing behaviour of the same neurone model was *modified* after implementing the features of AHP summation and depression. Firing behaviour thus predicted was also found in real DSCT neurones, most notably the two phases of firing acceleration given by the AHP depression. The steady state f/I relation of the model was *linear*, as demonstrated earlier for DSCT neurones (Eide et al 1964). It was concluded that also in DSCT neurones the AHP is of decisive *importance* for the firing regulation.

8 The model based on the AHP conductance thus *not only* has met with the constraints imposed from previous studies on *repetitive* firing in motoneurons (Kernell 1965 c, Schwindt and Calvin 1972, *in Kernell* 1973) but also predicted firing behaviour subsequently *confirmed* in real neurones. However it was recognized that the *model* is insufficient in accounting for several aspects of the firing *behaviour* in real neurones. Additional mechanisms such as membrane *non* linearities, sodium conductance inactivation, sodium electrogenic *processes* and the neuronal geometry are discussed.

9 The descriptive terminology for the trajectory behaviour (Schwindt and Calvin 1972, Schwindt 1973) and *for* f/I relations (Kernell 1965 c, Schwindt 1973) are discussed in relation to the model behaviour. It is concluded that this terminology in some respects is inconsistent and can give rise to misinterpretations of underlying mechanisms.

10 The results in the present investigation are discussed in relation to findings in other central neurones. A distinction in AHP properties and firing behaviour between neurones with and without connexion to muscles is recognized and the difference is discussed.

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SUPPLEMENTUM 417

Physical Properties of Connective
Tissue as Influenced by Single
and Repeated Pregnancies in the Rat

ÅKE RUNDGREN

GÖTEBORG AND ÅRHUS 1974

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 417

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BY
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GENERAL INTRODUCTION

From the standpoint of physiological function, pregnancy cannot be regarded as a process of fetal growth superimposed on the ordinary metabolism of the mother. The development of the fetus is accompanied by extensive changes in the body composition and metabolism of the mother. Many of the adjustments begin in early pregnancy before fetal growth is appreciable and therefore cannot be regarded as reactions to "stress" from the growing uterus and its contents.

Much attention has been paid to maternal alterations of the blood volume and composition, cardiovascular dynamics, renal function, alimentary function and water and electrolyte metabolism. On the other hand little interest has been focused on functional aspects of the connective tissues, although they constitute the "skeleton" of all soft tissues. Most studies of connective tissues during pregnancy are done from a biochemical point of view and studies of functional properties have mostly been limited to the genital tract, especially the uterus.

The purpose of the present investigation has been to attempt to at least partially answer the following questions:

1. How do the physical properties of the collagenous framework of the reproductive organs change during the different phases of pregnancy and are these changes due to quantitative or qualitative alterations?
2. Are there alterations in the physical properties of the extragenital connective tissue during pregnancy?
3. Are the maternal changes of both the genital and extragenital connective tissue completely reversible or will repeated pregnancies result in altered physical properties compared to virginal animals of the same age?

The connective tissue

Connective tissues contain cells and an extracellular matrix with the latter dominating and consisting of fibers, mostly collagenous but also elastic and reticular and ground substance. The proportions and arrangement of the fibers and the composition of the ground substance vary with the particular type of connective tissue.

The collagenous framework

Collagen the principal fibrous protein on which the physical properties of the connective tissue largely depend, constitutes approximately one third of the total body protein. It is present in phylogenetically very old invertebrate species (Pikkarainen and Kulonen, 1969) and, at least among mammals, exhibits little or no variation between species (Piez and Gross, 1959).

The synthesis of collagen has been reviewed by Udenfriend (1966) Gould (1968) and Rosenbloom and Prockop (1969). A number of mesenchymal cells, not only fibroblasts, osteoblasts and chondroblasts, but also smooth muscle cells (Ross and Klebanoff 1971) can synthesize collagen. The collagen molecule consists of about 330 residues of glycine, 124 of proline, 93 of hydroxyproline and 110 of alanine per 1000 amino acid residues (Pikkarainen, 1968). It is formed from amino acids into three polypeptide chains in the endoplasmic reticulum and is deposited in the extracellular space. In the collagen molecule from tissues such as skin and bone two of the chains are identical and the third differs slightly in amino acid composition. However in collagen from cartilage the molecule is assembled from three identical chains (Strawach and Nimni, 1971). The polypeptide chains are coiled into left hand helices and the molecule itself is a right hand super helix made up of the three polypeptide chains (Rich and Crick, 1955). The entire structure is held together by intramolecular bonds which can be broken by heat or by concentrated electrolyte solutions (Banga et al. 1956; Elden and Boucek, 1962). When the molecular structure is thus broken the individual chains are found as randomly coiled structures. The physical characteristics of the molecular structure have been deduced from physicochemical studies, X ray diffraction analysis and electron microscopy. The molecule is a rod-like particle approximately 300 nm in length, 1.5 nm in diameter and has a molecular weight of about 300,000 (Piez, 1967).

The process of fibrogenesis has been reviewed by Wood (1964) and Fitton Jackson (1968). The collagen molecules aggregate into fibrils, but the mechanisms behind this are unknown. Native collagen is formed as cross-striated fibrils of unknown length. The diameter of these fibrils varies depending on the sources of the collagen and the age of the animal (Greenlee and Ross, 1967; Wasserman, 1956). The axial periodicity of the fibril as determined by electron microscopy and small angle X ray diffraction is 64 nm in the dry state and 68 nm in the wet state (Bear 1944; Virdik and Ekholm, 1968). The length of the collagen molecule is 44 times the native fibril period. It has been suggested that the discrepancy between the native period and molecule length may be explained by a combination of overlaps and gaps between the molecules (Hodge and Petruska, 1962; Olsen, 1963; Smith, 1968). This implies that the cohesive forces act laterally and that there are no end-to-end attachments between the molecules. Collagen fibrils progressively assemble to form fibers 1-12 μm in diameter visible in the light microscope. The process of aggregation is accompanied by a change in the solubility of the collagen. Collagen molecules aggregate into fibrils that are soluble in neutral salt solutions. The randomly arranged newly formed fibrils are organized into a more complex fiber meshwork and with time the solubility gradually decreases as a result of increased cross-linking.

The mammalian tendon consists almost entirely of bundles of fibers lying approximately parallel to its long axis. The fibers are presumably as long as the tendon (Bucher 1962). In more complicated patterns of the collagenous framework other than the tendon, the length of the fiber probably varies considerably.

There is no specific histologic staining method for positive identification of collagen, although there is a correlation between staining properties with for example van Gieson, Mallory and Masson methods and the presence of collagen established by other methods. However the absence of staining does not exclude the presence of collagen (Harkness, 1961). Birefringence is exhibited in polarized light and is positive in the direction of the longitudinal axis of the fibril.

Ground substance

The collagen fiber framework is closely associated with the amorphous ground

substance which is formed by the same types of cells as collagen molecules and is secreted into the extracellular space. In dense connective tissue such as tendon the amount of ground substance is small, but it is a dominating component in loose connective tissue. This can be shown in electron microscopy using the freeze-etching technique. This method retains the ground substance thereby providing a much better mean of evaluation of this component than is possible in thin sections from plastic embedded tissue. The main components of ground substance are the acid mucopolysaccharides or glucosaminoglycans, the chemistry of which have been reviewed by Muir (1964) Gardell et al. (1969) and Laurent et al. (1969). Most if not all of the mucopolysaccharides in the connective tissue are linked to proteins or peptides by covalent bonds. The mucoproteins and mucopolysaccharide proteins take an integral part in stabilizing the collagen fibers by being firmly bound to the fibrils (Mathews, 1965). However what significance this might have for the rheological parameters in connective tissue is not known (Vidlik, 1973). The ground substance is a hydrophilic gel (Chrisman, 1964) which binds water (Ogston, 1966) and the water content influences the physical properties of connective tissue (Galante, 1967).

During aging changes in the composition as well as the concentration of mucopolysaccharides in the connective tissue have been reported. It is probable that these chemical changes are accompanied by changes in the physical properties of the macromolecules which in turn would influence the properties of the tissue (Gardell et al. 1969).

Turnover and catabolism of collagen

The metabolic turnover of collagen is very slow especially in old age (Thompson and Ballou 1956). The liver and bone collagens have the highest rate of turnover while collagen in tendons has a very long half life (Neuberger 1965, Woessner 1968). There are normal and pathologic states of some tissues in which collagen turnover or breakdown is high. Examples of the former are found in the remodelling of bone during growth and the partial resorption or restoration of the uterus after parturition. The mammalian uterus is a very favourable tissue for the study of collagen catabolism under normal physiological conditions because during the course of involution which follows parturition, there is an exceedingly rapid breakdown with little concomitant

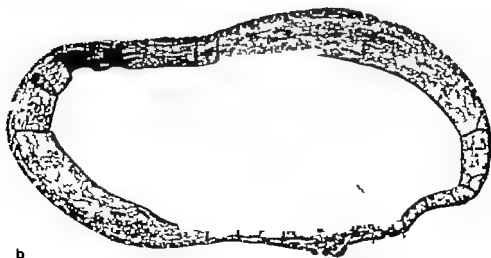
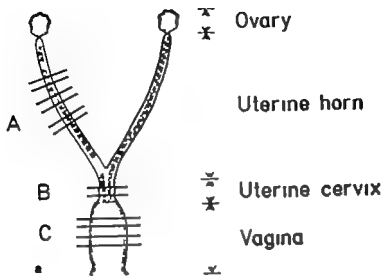
collagen synthesis (Woessner 1962) In a number of bone diseases and hyperthyroidism there are elevated levels of peptide bound hydroxyproline in plasma and urine (Prockop and Kivirikko 1967) In these states and at the particular site involved a collagenase may be present. There are, however only a few sources from which an enzyme can be prepared that will hydrolyze native collagen fibers near pH 7 The concept of collagen breakdown involves tissue collagenase as well as lysosomal enzymes, specifically cathepsin D (Eisen et al. 1970) but it is not clear whether these enzymes attack collagen together at the same site or at independent sites of the molecule.

Geometry of connective tissue

The arrangement of the collagenous framework is quite varied and depends on different functional demands. The geometrical pattern of dense connective tissue can be divided into (1) more or less parallel-fibered arrangements, (2) two- and three-dimensional mesh works and (3) complicated patterns where interaction with other elements occur i.e. elastic elements, smooth muscle cells and crystalline solids (Vidlik, 1973)

(1) *Parallel fibered structures.* The tendon is considered to be the most regular parallel-fibered structure. Tendons contain about 30 per cent collagen by wet weight but about 70-80 per cent by dry weight. Elastic and reticular fibers are very scarce. The fibrils are packed closely and parallel to each other making up the tendon fibers which are assembled into fiber bundles. In the relaxed specimen bundles of fibers display a slight waviness (Rigby et al. 1959 Vidlik and Ekholm, 1968) The bundles, which are surrounded by sheaths, fuse with each other at acute angles as do the fibers (Vidlik, 1973) Fibrocytes are present in long parallel rows between the bundles. A number of bundles are bound by loose connective tissue into large bundles and finally into the complete tendon. Outside the tendon is loose, areolar tissue.

A joint ligament has a similar somewhat less regular parallel-fibered arrangement which is due to differences in functional properties. In ligaments the fiber course is often partly oblique or spiral. Such geometrical arrangements have importance for the mechanical behavior of ligaments, i.e. the degree of straining of the various fibers of the ligament varies (Vidlik, 1973) Most ligaments consist of pure collagen having elastic fibers only accompanying the blood vessels. A ligament has both its ends inserted into bone, but the



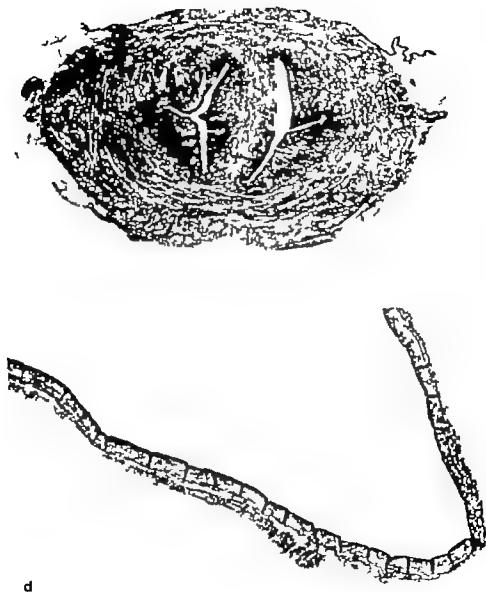
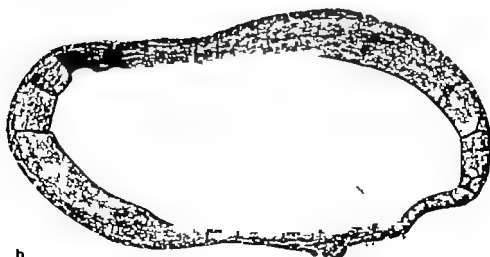
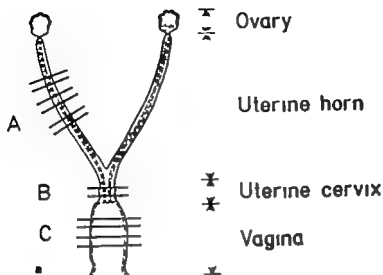


Fig 1 The genital tract in the rat. Schematical picture () with A, B and C indicating the specimen sites. Microphotographs (21 x) of cross-sections of (b) uterine horn () uterine cervix (d) and vagina. The specimens were fixated with formalin under intraluminal pressure.



fiber arrangement in the symphysis of the rat is mainly an intermingling cross-cross pattern of fibers in the cranio-caudal and dorso-ventral planes. No transversely directed fibers are found (Crelin and Brightman, 1957). Thus the collagenous framework in the pubic symphysis has a complicated three-dimensional structure where the length of the fibers varies considerably. This fact might to some degree explain the observed differences of the load-deformation curve for the pubic symphysis compared to that of the posterior cruciate ligament (Cf. Part II).

Physical properties of collagen

The physical properties of collagen are dependent on the arrangement of the fibers and in particular on the bondings between the components at levels from the molecules to the fibers. Cross-linking of collagen molecules is a normal step in development and is essential for the formation of strong collagen fibers and normal connective tissue (Levene and Gross, 1959; Gross et al. 1963).

When collagen fibers are heated in the presence of water or are exposed to strong solutions of electrolytes they contract (Verzár 1955). Contraction takes place when the thermal energy is sufficient to break up the ordered structure to a state of random disorder. Covalent cross-links are not affected, only hydrogen bonds and salt-like cross-links are disrupted (Jackson, 1965). In isometric contraction old tissues develop higher tensions than young ones (Verzár 1963).

Changes in collagen noted with increasing age are considered to be a part of the normal growth and development and are designed to fulfill the need for the bulk of collagen to be insoluble and metabolically inert for optimal function as part of supporting structures (Jackson, 1965). However in mature fibers Mohanaradhakrishnan and Ramanathan (1963) found that, although an increase of the extent of cross-linking with tanning agents increased the shrinkage temperature, this treatment had the opposite effect on the breaking strength. It would thus seem that there is an optimal number of intermolecular cross-links required to achieve maximal strength, and that an increase in the number of cross-links beyond that point has an adverse effect on the strength.

Biomechanical properties

Mechanical properties of connective tissue are those most clearly related to the function, and information about these can be obtained by biomechanical analyses. A force is required to alter mechanically a specimen of a material. When changes in shape or tension or both are studied the specimen must be fixed to a point at which a counterforce exists that equals the test force. When an object is fixed by one of its ends in a fixture and loaded by means of a weight applied to its mobile end, a tension is created in the specimen. The mass of the weight is measured in grams or kilograms and the tension created between the two ends of the specimen is expressed in newton or in kilopond (the force of 1 kp = 9.81 N = 2.20 lb; is created when suspending the mass of 1 kg). If the load is large enough the distance between the two ends of the specimen increases resulting in a deformation. This is dependent on the length and cross-sectional area of the specimen. If the material and not a specific specimen with its individual geometrical characteristics is to be investigated, the load is expressed per unit cross-sectional area and is called stress. The original length together with the mechanical properties of the specimen determines how much the specimen is elongated, and therefore the length change is expressed in units of original length and is called strain. If the relationship between stress and strain is linear i.e. the material is perfectly elastic, this can be expressed as a ratio (e.g. N/mm²) the modulus of elasticity.

The ultimate tensile strength of a material indicates how much it can be stressed before it fails. There are, however, points before this value is reached which are of interest because no material is perfectly elastic. For soft connective tissues the load-deformation or stress-strain curve (Fig. 4) starts with a toe-part with the concavity towards the load or stress axis. This corresponds to a straightening of the initially wavy formation of the collagenous bundles in tendons (Rigby et al. 1959; Viidik, 1973). With increasing stress the fibers will be arranged in the direction of the load corresponding to a fairly linear section on the curve (Viidik, 1968a). The rupture may occur in this region (Morgan, 1960; Viidik, 1968a) or the curve may bend off towards the deformation axis before rupture occurs. The load value may even decrease below the maximum value before complete failure (Viidik et al. 1965).

The load-deformation and stress-strain curves for collagenous tissues are

sigmoid in shape, and thus the concept modulus of elasticity based on a linear relationship between load and deformation cannot be used. However for the fairly linear part of the curve the tangent value for the angle between the deformation axis and the slope of the linear region can be measured. This is here called "elastic stiffness" for a load-deformation curve and "modulus of elasticity" for a stress-strain curve. These parameters thus indicate the stiffness of the specimen and material respectively for the part of the curve following the toe-part. Many investigators in biomechanics have analysed only the ultimate tensile strength. For collagenous tissues this is reached only when considerable external force is applied. Even then, most often some other part such as the insertion into bone fails before the tendon or the ligament. The analysis should be extended to obtain the abundant information that is offered by the stress-strain curve, especially within the normal functional range of the tissue.

The mechanical properties of connective tissue can be described with a mechanical analogy. It has not been possible to formulate a perfect equation for the visco-elastic behaviour of this tissue because there is a high degree of non-linearity in the load-deformation relationship. Viidik (1968d) proposed a mechanical analogy for parallel fibered collagenous tissue from experiments on knee joint ligaments in rabbits. Based on the analogy a mathematical equation was formulated from which the mechanical properties under certain testing conditions could be predicted, and these were verified experimentally (Frisén et al. 1969a, 1969b).

Basic materials and methods

Animals

Young pregnant rats. 98 female Wistar rats of the same strain, aged 150-170 days, were used. The estrus cycle was followed by vaginal smears (Fig. 2). When the smears showed homogenous, nucleated epithelial cells without leucocytes the animals were mated for 12 hours. Then a new smear was made and if spermatozoa could be seen the animal was considered to be in the first day of pregnancy. At the time of testing all non pregnant rats were in the diestrus phase. For the rats that were not sacrificed in the gestational period the time of parturition was recorded and the animals were allowed to nurse.



Fig 2 Vaginal smears of a young rat. (a) diestrus phase (b) estrus phase of the estrus cycle (105 x)

their litters. The animals had free access to food and water and were housed under constant conditions of room temperature, air humidity and light.

Old multiparous rats 38 Wistar rats of the same strain as above were investigated at the age of 22-23 months. The animals, except for 9 controls, were mated between the 4-17th months of age. The litters were nursed for three weeks. No pregnancy occurred during the four months before the experiment. A birth record for each rat was carefully kept with regard to date of parturition and size of litter. After the fertile period the animals were divided into three groups consisting of those with 4, 5 or 6 pregnancies. The animals had free access to food and water and the housing conditions were as noted above. The original number of rats was 58 but during the 17-18 months of observation 20 animals died. During the last four months the number of deaths was seven and of these 3, 2 and 2 belonged to the 6, 5 and 4 pregnancies groups respectively. All rats in the control group survived during the observation time.

Table I summarizes the groups of the young and the old rats, the number of animals and mean body weight for each group.

At the time of the experiment the animals were sacrificed by an overdose of ether and the various organs carefully dissected. The specimens, if not tested immediately, were stored in buffered Ringer's solution, pH 7.4 (genital organs) or in saline moisten gauze (pubic symphysis and the intact hind limbs con-

Table 1 Body weights of the young and old rats used in the present investigation.

	N of animals	g
YOUNG		
Control	16	261 \pm 7
Gestational		
Days		
6-10	3	281 \pm 13
13-14	8	298 \pm 15**
15-16	9	291 \pm 12**
17-18	7	310 \pm 12**
19-20	11	318 \pm 7**
21	4	329 \pm 11**
Post-partum		
Days		
1	5	281 \pm 6**
2	2	270 \pm 4
3-5	8	274 \pm 12
7-9	2	267 \pm 4
12-14	7	294 \pm 4**
15-16	4	288 \pm 8**
17-20	6	287 \pm 7**
23-38	4	283 \pm 5**
OLD		
N of pregnancies		
0=control	9	315 \pm 10**
4	15	359 \pm 12**
5	10	360 \pm 18**
6	4	371 \pm 37
4+5+6	29	356 \pm 10**

** 2P<0.05 against young control.

** 2P<0.05 against old control.

taining the muscle tendons and posterior cruciate ligaments) for a period usually less than two hours at room temperature.

Testing equipment

The materials testing machine is shown in Fig 3. It consists of a horizontal

metal frame with the load transducer (Bofors KRK 1 50 N) at the fixed end. The mobile end was operated via a backlash-free screw arrangement (according to Vildik, 1966) and driven by an electric motor the speed of which was controlled by a thyristor unit. The movement was recorded by a displacement transducer of differential transformer type (Bofors, RLK 1-S ± 6 mm). The clamps to which the specimen was mounted were fastened to the fixed and mobile ends of the system. The deformation speed was kept constant at 10 millimeter per minute. Each transducer was coupled to a direct reading measuring bridge (Philips PT 1200) and the resulting load and deformation d.c. signals were fed to the X and Y inputs of a Hewlett Packard Model 7004A ink recorder. The clamps with the specimen were placed horizontally in the testing machine (Fig. 3) and balanced by thin steel wires until the specimen, clamps and transducers were in the same plane. By this procedure influences of vertical forces on the load readings were avoided. The error in the measuring system was estimated for load by multiple loadings of the load cells with known weights and for deformation by displacing the iron core of the differential transformer with the aid of a micrometer. It was in all instances found to be in the range of 0.2 - 0.7 per cent of maximum signals in all measuring ranges.

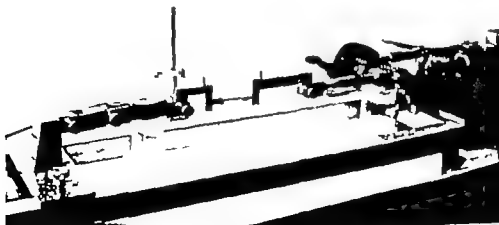


Fig 3 The materials testing machine. The force transducer is seen to the left. The clamps are in the middle of the picture and to the right the displacement transducer and part of the backlash free screw arrangement are seen. The motor is situated outside the picture to the right.

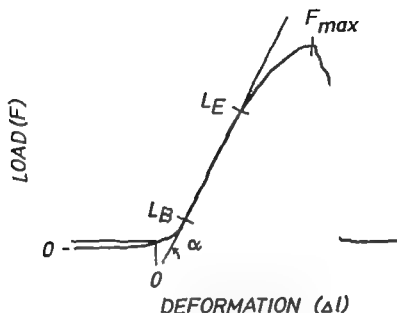


Fig 4 A schematic load-deformation curve for collagenous tissue. L_B = begin of the linear region L_E = end of the linear region F_{max} = point of failure, α = angle between the linear region and the deformation axis elastic stiffness

Experimental techniques

Parameters

A schematic load-deformation curve for a parallel fibered collagenous tissue is shown in Fig 4. The following parameters were calculated from the curves: (1) load (F) and deformation (Δl) for the start of the linear region of the curve, L_B , (2) the end of the same region, L_E , (3) the point of maximum load, F_{max} and (4) the "elastic stiffness" expressed as the tangent, $\tan \alpha$, for the angle of the linear region of the curve and the X -axis. The figures of the various parameters were read from the XY recorder charts to the nearest 0.25 mm. The $\tan \alpha$ values were calculated on the basis of the X and Y coordinates of L_B and L_E .

Load and deformation were expressed in newton and millimeter respectively. Except for the pubic symphysis and posterior cruciate ligament specimens the load and deformation were also calculated for stress (σ) and strain

(e) For each specimen the load values were normalized to units of collagen (i.e. mg collagen per millimeter specimen length) giving the stress dimension $N/mg/mm$, and the deformation values were normalized to the original length (l_0) giving the dimensionless strain. By this procedure qualitative differences in the specimens can be analysed. The original length (l_0) is defined here as the length of the specimen when it starts to resist loading, which point was considered to be reached after a two millimeter change in the Y-direction on the recorder chart (at standard calibration for specimens with the highest failure load) corresponding to about 0.08 N

Chemical analysis

After the mechanical test the collagen content in the specimen except for the pubic symphysis and posterior cruciate ligament, was determined by quantitative hydroxyproline analysis according to the method of Stegeman (1958) as modified by Bergman and Loxley (1963). The specimen was hydrolyzed in 6N hydrochloric acid at 130 C for three hours in sealed Pyrex® tubes. Chloramine-T was added to oxidize hydroxyproline to a pyrrole compound which in combination with p-dimethylaminobenzaldehyde yields a red color. The red color was measured spectrophotometrically at 568 μm (wave length). The collagen content was calculated as 7.46 times the hydroxyproline content (Neuman and Logan, 1950).

Statistical methods

The load-deformation curves were analysed for the parameters presented above. When more than one specimen from an organ in the same animal was tested, the mean values for the individual animals were used to calculate the mean value of the group. For each group mean value and standard error of the mean were calculated and various groups were compared with the control group by Student's *t* test. Differences were regarded as significant if $2P < 0.05$ and tending to be significant if $2P < 0.10$ (two-sided test).

Remarks on experimental methods

The techniques used by investigators to evaluate various mechanical properties of connective tissue have varied to a great extent. Most of the testing proce

dures come from the field of materials strength testing, but the methods cannot be adopted without modifications and proper attention to the peculiarities of the biological material. The collagen fiber never functions isolated and is always part of a functional unit as in the locomotive system in a bone-tendon-muscle-tendon-bone or bone-ligament bone complex. Testing the whole complex gives the performance of the collagenous structure to the failure of the weakest point in the system, which never is in the tendon or ligament (Vidlik, 1968a). For elucidation of the full range of the properties of the tendon or the ligament it must be tested isolated. There are, however many problems connected with this.

In whole functional units the bones are the natural point for fixation preferably by clamping. Testing of isolated tendons creates many problems, especially with regard to adequate clamping. Often the results are distorted due to slipping of the specimen or breaking in the jaw of the clamp. Fresh material has low rigidity and when clamped tends to "flow". As will be described in Part II the tendon and skin specimens broke at one of the jaw edges. When the specimen starts to fail, a deviation of the curve towards the strain axis ending the linear region is noted. This point and the breaking point are influenced by the clamping procedure, while the coordinates of the start of the linear region and the "elastic stiffness" should be unaffected by this. Jaw breaks were also encountered by Wright and Rennels (1964) in their study of human plantar fascia. They noted that the failure took place somewhere in the linear region of the curves. However conclusions about both the "toe-part" and the "modulus of elasticity" could be made.

When evaluating the tensile properties of geometrically non-congruent specimens it is of importance to know the cross-sectional area. Many difficulties are encountered due, for example to variation in the cross-sectional area throughout the length of the specimen. This and other factors might account for differences in results reported on tensile strength measurements. Tensile strength determinations were well correlated to the values of fresh weight per unit length for tendons from animals of uniform functional state and age (Vidlik, 1967). By this technique a measure corresponding to a mean value for the cross-sectional area of the whole tendon was obtained instead of values at arbitrary measuring points. The amount of collagen per unit area or unit length of the specimen proved to be an adequate parameter (Fry et al. 1964 Vidlik, 1970). This type of measurement was selected for the

assessment of the functional cross-sectional area in the present study because the genital organs and skin have considerable amounts of ground substance and other tissue components in addition to collagen that add to weight but not to strength. The "stress" values are thus load values normalized to mg collagen per mm specimen length. This method was also applied to the muscle tendons since it had been shown to be satisfactory in biomechanical tests of the chordae tendineae (Vidlik, 1970)

The other basic parameter to measure is the original length. For parallel fibered collagen specimens it is advantageous to measure the length of the specimen between the clamps after a small but safely recordable stress, corresponding to one to two per cent of the failure value is applied. This makes certain that the specimen is straightened in a standardized way. When testing a bone-ligament-bone system such as the posterior cruciate ligament of the knee joint and the bone fibrocartilage bone system of the pubic symphysis, the measurement of the original length as well as that of the cross-sectional area becomes almost impossible without damaging the specimen. The insertions of the ligament are fairly broad and the thickness varies being least in the middle. In the pubic symphysis of the rat the width varies along its coronal plane being about one millimeter in the cranial and midsections and somewhat wider caudally. The ventral portion of the articular capsule is thick due to the origin of the fibers of the rectus abdominus muscle and obscures the bone-cartilage junction. By disregarding the original length the testing will be qualitative rather than quantitative, but it is fair to assume that the various specimens are reasonable congruent in the same stock of animals of the same age (Vidlik et al. 1965 Vidlik, 1968c Crelm and Brightman, 1957)

The measurement of the original length in specimens with a lumen such as the genital organs presents another problem. The wall of the specimen in the unloaded conditions is approximately evenly thick. During loading there will be a certain "flow" from the points where the force is applied to the part of the specimen that are not in the line of the force application. The potential source of error for deformation or strain measurements lies in this behaviour of the specimen and will be greater the thicker the wall. Harkness and Harkness (1959a) considers the uterine cervix as a belt and ignored the complications due to the thickness of the wall. They used the inner circumference as the original length measurement after the specimen was loaded for 5 seconds with 0.5 N. By applying this load the collagenous framework is

probably stretched and the value will not represent the true original length. In the present investigation the ring formed specimen is considered as a solid body. The "flow" of tissue during loading is disregarded as there is no simple method to measure it. The original length of the specimen is measured on a photograph as the distance between the mean of the inner and outer walls at each of the two force application points in the completely unloaded specimen. To this value is added the distance in the recorder chart from the start of the recording to the point on the curve where a change in Y-direction safely has been recorded. This was usually equivalent to about 0.08 N with a little variation due to small changes in the Y-axis calibration.

Testing tendons, ligaments and most other collagenous tissues in completely intravital conditions is impossible. Attention must thus be paid to possible post mortem changes. The water content of the tissue is important because drying increases the stiffness of the tissue (Galante, 1967). Other post mortem changes are less evident. No changes were observed in various tensile strength parameters when storing intact knee joint specimens for as long as 3 days (Viidik et al. 1965, Tipton et al. 1967). This is also in agreement with the results of experiments performed on skin (Ridge and Wright, 1965). For refined rheological analyses it is important to use standardized procedures because post mortem osmotic, chemical and metabolic changes may influence the results (Tipton et al. 1972). Collagenous tissue swells in water (Gustavsson, 1956). This is also true for "physiological" saline (Viidik and Lewin, 1965) and the tissue exhibits subsequent changes in its mechanical properties after immersion in this solution. The magnitude of swelling in a tissue is also related to its degree of organization and the amount of internal stabilization of its proteins (Gustavsson, 1956). A gelatin gel with the same protein content as a piece of skin exhibits much greater swelling than the skin (Galante, 1967). Exposure to air causes progressive water loss from the tissue and is greatest when first exposed. The most effective methods to keep the tissue from drying is to keep it in humidity of 100 per cent (Hirsch and Galante, 1967) or to wrap the structure in a saline moistened gauze (Viidik and Lewin, 1965). The latter technique prevents changes in the mechanical properties for at least 4 hours. By using this technique or immersion in trisbuffered Ringer's solution with a constant pH of 7.4 when necessary the environmental factors are standardized and inter-experiment variation minimized in this series of experiments.

Part I

BIOMECHANICAL PROPERTIES OF THE REPRODUCTIVE TRACT

Introduction

The collagenous part of the connective tissue has a low rate of metabolism in most organs (Neuberger and Slack, 1953). However in the reproductive tract, during a short period of time, the collagenous framework can adapt itself to new functional demands imposed by gestation and parturition and subsequently restore itself in the following involution period. The rapid synthesis and laying down of the different connective tissue components during gestation and the even more rapid post partum breakdown change the functional properties, as have been shown by earlier biomechanical investigations (Harkness, 1964; Harkness & Harkness, 1959a, 1961, 1965).

A part of the reproductive tract of special interest from a connective tissue point of view is the uterine cervix because of its high collagen content and the extreme requirements placed on its functional behavior at the time of parturition. Biomechanical investigations of the uterine cervix in rat have shown an increased extensibility of the collagenous framework during gestation (De Vaal, 1946; Uyidert and de Vaal, 1947; Harkness and Harkness, 1959a, 1961; Zarrow and Yochum, 1961). The physical properties of the uterine horns have been studied by Harkness and Harkness (1965) who found extensibility unchanged during pregnancy and increased ultimate tensile strength at parturition. Less attention has been devoted to the vagina where the morphological changes during pregnancy are not so conspicuous. Changes in physical properties of the vaginal wall do, however occur (Harkness and Harkness, 1965) but information is available only for a few time intervals during gestation and post partum period.

The purpose of this part of the present investigation is (1) to follow the biomechanical properties of the genital organs during the different phases of a single pregnancy and the subsequent involution, (2) to analyze these

organs for the same properties after repeated pregnancies and (3) to estimate the effect of aging on the physical properties of the genital tract by comparing young and old virgin animals. When analyzing the biomechanical parameters correlation has been made to changes in the collagenous framework.

Uterine horns

Young pregnant rats. A large part of the endometrium in the uterine horns in the rat consist of connective tissue lying beneath the epithelium. The endometrial collagen fiber mesh work in virgin rats consists mostly of coarse bundles but during pregnancy the larger fiber bundles are transformed into a network of small anastomosing fibers and the amount of ground substance increases (Faust, 1964). The myometrium has one inner circular and one outer longitudinal layer of smooth muscle fibers separated by scant amount of connective tissue. Just above the uterine cervix the smooth muscle cells constitute 40-50 per cent of the total tissue volume of the non-pregnant rat as estimated by histological methods (Harkness and Harkness, 1959a).

The estrus cycle changes the wet weight of the uterus considerably with a maximum of 128 per cent at estrus and a minimum of 84 per cent at diestrus based on the mean values for all stages but does not influence its collagen content (Harkness et al. 1957). The collagen concentration in the horns of non-pregnant rats at estrus is 2.8 per cent of the wet weight and decreases to 2.2 per cent at the end of gestation (Harkness and Harkness, 1954). Of the total protein content of rat uterus the collagen constitutes 30 per cent in the non-pregnant state and 20 per cent during late gestation (Montfort and Pérez Tamayo, 1961).

During gestation in the rat the wet weight of the uterus increases about seven times and the total collagen content six times, but the increase in both these parameters is slight until the 12th day of gestation, when a rapid growth ensues (Harkness and Harkness, 1954). During gestation in an empty horn secondary to ovariectomy on one side, some growth of collagen takes place in the first half of pregnancy (Harkness and Harkness, 1956; Harkness and Moralez, 1956). While the horn containing fetuses continues to grow the empty horn remains unchanged for the rest of the gestation. There is also evidence that distension of the uterine horn with wax increases the amount of collagen (Cullen and Harkness, 1959). After parturition the wet weight

and collagen content decrease to a level about 50 per cent below the values of virginal animals of equal age (Harkness and Moralec, 1956 Woessner and Brewer 1963) During the early post partum period abundant macrophages with conspicuous enzymatic activity probably take part in the degradation of ground substance and fibrillar elements (Lobel and Deane, 1961) and fragments of collagen fibrils have been found by electron microscopy in these cells (Brandes and Anton, 1969) Increased concentrations of proteolytic enzymes in the uterus during the involution have been reported (Woessner 1962 1968 Woessner and Brewer 1963 Schaub 1963/66) Jeffrey and Gross (1970) were able to partly isolate and characterize a collagenase from involuting horns and Ryan and Woessner (1971) demonstrated collagenase activity from similar tissue.

Reports concerning elastin in the uterus are much more scarce than about collagen. Histological investigations have shown that most of the elastin is in the walls of the blood vessels, although elastic fibers can be seen throughout the myometrium (Albert, 1966) Chemical studies on human uterus indicate that during pregnancy the amount of elastin increases proportionally as much as collagen about five to six times and decreases during the post partum period (Woessner and Brewer 1963)

The ground substance of the uterus has mostly been studied by histological techniques. As judged by these investigations there is an increase in the ground substance of the uterine connective tissue in rat during gestation (Maibenco 1960)

Investigations of physical properties of the uterus have been concerned more often with the smooth muscle cells than the connective tissue (Scapo and Goodall 1954 Zimmer 1959) Harkness and Harkness (1965) determined the ultimate tensile strength and "extensibility" (i.e. rate of specimen extension at a constant load) in ringshaped specimens from uterine horns from virginal gestational and one day post partum rats. The tensile strength expressed in N/mm^2 (cross-section area calculated to be pure collagen) was 13 on the 12th day of pregnancy and reached 29 on the 21st day of pregnancy 24 hours post partum this value was reduced to 12, approximately the same as that in virginal animals. There was no evidence of change in "extensibility" at parturition while the uterine cervix showed very high values for this property at this time.

Old multiparous rats Morphologic studies on the effect of repeated preg

nances in intact and unilaterally ovariectomized mice have been performed by Biggers et al. (1962) who found the functioning uterine horn in the operated animals hypertrophied. This shows the importance of mechanical distension caused by the fetuses as a growth stimulus since the hormonal influence was the same for both the active and inactive horn. Finn et al. (1963) repeated the investigation and determined the collagen content in the horns of mice at the age of 24 months. They found the difference between the two horns negligible. No old virginal animals were, however included in the study. Compared with two month old virginal animals the collagen content in the uterine horns had increased three times. The increase in the collagen content of whole uterus in virginal rats is negligible after early adult life (McGavack and Kao, 1963). Maurer and Foote (1972) reported that three to four days after parturition in multiparous rabbits of different age and number of previous pregnancies, the uterine collagen content tended to increase and the specific collagenase activity decreased with the number of pregnancies. No virginal animals were included in the investigation. Burack et al. (1941) studied by light microscopy the connective tissue in the reproductive tract of rats from young to old age. In virginal animals the growth of connective tissue was greater than in those with previous pregnancies and contributed to an increased thickness of the wall of 30 per cent compared to highly fertile breeders. In a biochemical investigation on biopsy materials from multiparous women Woessner and Brewer (1963) found an increase in the wet weight, the amount of collagen and the amount of elastin in the uterus compared to virginal females.

Uterine cervix

Young pregnant rats The uterine cervix of the rat, compared to other parts of the reproductive tract is a rather compact structure that can be easily distinguished although its transition to the uterine horns is somewhat gradual (Harkness and Harkness, 1959a). Histologically the connective tissue represents 50-65 per cent of all the tissue in the cervix. The smooth muscle cells are sparsely represented in the portio but increase cranially (Harkness and Harkness, 1959a).

The wet weight of the cervix in the rat varies with the estrus cycle but the dry weight does not (Datta et al. 1968). During gestation the increase in wet

weight starts after the first half of gestation. The increase of collagen parallels in time that of the wet weight being about doubled at parturition (Harkness and Harkness, 1959). At the time of parturition hexosamine is 2.5 per cent of dry weight compared to non pregnant rats, which have 0.5 per cent. The walls of the cervix are edematous and the bundles of collagen fibers are separated from each other by the abundant ground substance (Storey 1957, Leppi, 1964, Bryant et al. 1968, Leppi and Kinnison, 1971).

During the post partum phase the wet weight is reduced approximately eight times and the collagen content four to five times (Harkness and Harkness, 1961). While the wet weight reduction is noted almost immediately after parturition the catabolism of collagen is delayed for about 48 hours (Harkness and Harkness, 1959). Temperature variations as well as papaverine in were discussed above in connection with the uterine horns.

The ultimate tensile strength of the uterine cervix in the rat expressed in N/mm² collagen, is 13.2 at estrus and 9.8 at the 1st day of gestation (Harkness and Harkness, 1959a). Temperature variations as well as papaverine in the medium in which mechanical tests were performed do not influence the ultimate tensile strength except at parturition. At this time lower values were reported at 37 C than at 22 C. During parturition tensile strength measured at 22 C is 11.9. On the second day post partum it was 9.9 and on the 8th and 16th day 8.4 (Harkness and Harkness, 1961). The load-deformation procedure used by Harkness and Harkness (1959a, 1961) was a stepwise increase of load until the breaking point. Every 15 seconds a 0.25 N load was added, and before every increase of load the specimen was unloaded momentarily and the force on the tissue reduced to about zero.

The "extensibility" or deformation per unit of time of the uterine cervix during pregnancy was analysed by Harkness and Harkness (1959a, 1961). When studying this "creep" phenomenon the specimen was subjected to constant load for different periods of time. The cervix usually yielded rapidly in the beginning, but with time the specimen became more unyielding and the recorded curve reached an asymptote. At the time of parturition the "extensibility" was much more pronounced than during the rest of the pregnancy. This increased "extensibility" disappeared during the first day of the post partum period and values in the range of the control animals were recorded.

Old multiparous rats. Burack et al. (1941) studied by light microscopy the connective tissue of the uterine cervix in both virginal and multiparous rats

from young to old age. In virgins the growth of connective tissue was greater than in breeding animals and resulted in decidedly larger cervixes compared to breeders of the same age, but no actual measurements were reported. By the age of 20 months the stroma of the uterine cervix in virginal mice had become markedly fibrous or hyaline (Loeb et al. 1939). No information on biomechanical properties of uterine cervix in multiparous animals is known to the author.

Vaginal wall

Young pregnant rats. The vaginal wall in the rat is a highly collagenous structure with a collagen concentration of about 8 per cent of the wet weight in the non-pregnant animals during estrus (Harkness and Harkness, 1965). The histological picture of the connective tissue in the vaginal wall of young virginal rats is usually that of a closely interwoven collagenous meshwork in which a considerable number of fibroblasts are embedded and in which there is no distinct arrangement of the fibers (Barack et al. 1941).

Morphological changes in the vaginal wall of the rat during pregnancy have not been as thoroughly investigated as in man. Richin (1951) reported that in the non-pregnant human vaginal wall the connective tissue is of the loose fibrillary type and contains fairly numerous elastic fibers. This type of connective tissue is found in many places in the body. During gestation the connective tissue becomes looser due to increased amounts of ground substance as judged by histological methods. The larger collagen fibers found in non-pregnant individuals are not seen and instead small delicate fibers are identified.

Harkness and Harkness (1954) measured the collagen content and the wet weight during pregnancy in the rat. The weight increase about 50 per cent to the time of parturition, and during the involution phase it decreases towards the non-pregnant values. The total amount of collagen in the vaginal wall does not change during pregnancy but the collagen concentration decreases due to the weight increase. During gestation the circumference of the vaginal wall increases about 100 per cent to 50 millimeters compared to non-pregnant diestrus rats. The circumference at parturition is larger than the circumference of the fetal head which is reported to be about 37 millimeters (Harkness and Harkness, 1959a). One day after parturition this value

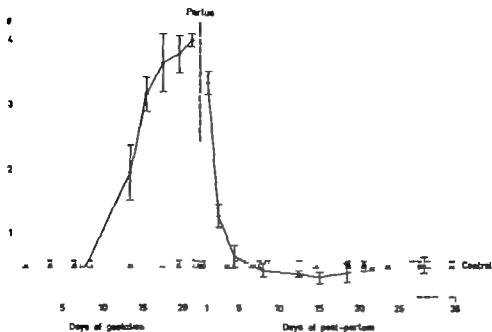


Fig 6 Wet weight of the uterine horns of young rats during pregnancy (Mean values \pm S.E.M.)

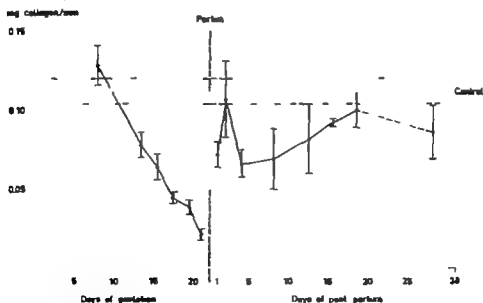


Fig 7 Amount of collagen per unit specimen length of the uterine horns of young rats during pregnancy (Mean values \pm S.E.M.)

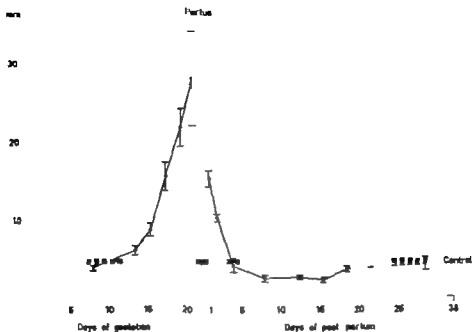


Fig 8 Original length (l_0) of uterine horns of young rats during pregnancy (Mean values \pm S.E.M.)

fifth of the value of the control group. During the post partum period there is a noticeable increase compared to the animals of late gestation. Due to rapid wet weight reduction and delayed collagen catabolism during the first days after parturition, the amount of collagen increases but then a slight decrease occurs. During late post partum there is again an increase towards the value of the control group.

The original lengths (l_0 = the length of the specimens when they start to resist force) are considerably increased during gestation (Fig 8) reflecting the distension of the uterine wall by the growing fetuses. The involution progresses rapidly during the early post partum period and after the fifth day the original length is less than in the virgin animals. In the late post partum period the values approach those of the controls again.

During the mechanical testings the specimens usually failed at the end near and lateral to the point where the clamp was in contact with the tissue. The results of the gestational and post partum uterine horn specimens from these

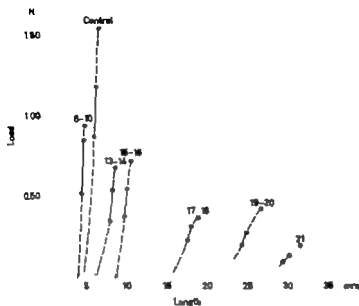


Fig 9 Mean load-length curves of uterine horn specimens of young rats during gestation. The figures of the tops of the curves denote days of gestation.

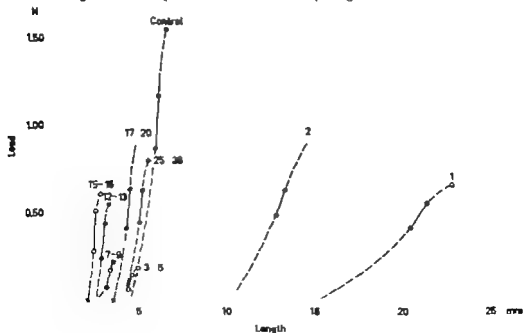


Fig 10 Mean load-length curves of uterine horn specimens of young rats during the post partum period. The figures of the tops of the curves denote days after parturition.

K/mg collagen/mm²
16.0

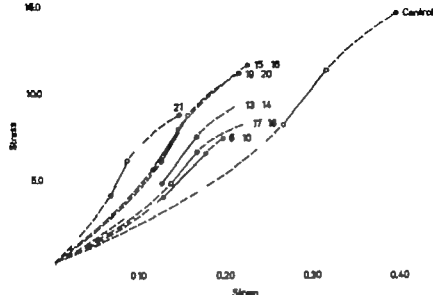


Fig 11 Mean stress-strain curves of uterine horn specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

tests are presented in Figs. 9 and 10 and Table A I (A=appendix). The load-deformation values are listed and curves for load versus length for the different groups show the relaxed length at zero load, after which the actual load-deformation curve begins. Due to the growth of the horns during gestation the curves move on the length-deformation axis towards larger values. There is also an increase in the total deformation of the specimen, 1.9 mm for the control and 4.8 mm for the 19-20 day group. The maximum load is by far greatest for the control group and during gestation shows a sharp reduction approaching parturition. The breaking load for the 21 day animals is only approximately 15 per cent compared to the controls. In the linear region of the curves the "elastic stiffness" also shows a decrease during gestation. Deformation per unit load increases considerably and is most conspicuous near parturition.

During the first two days post partum the load values increase but they are sharply reduced when catabolism of the collagen starts. The reduction is

N/mg collagen/mm

150

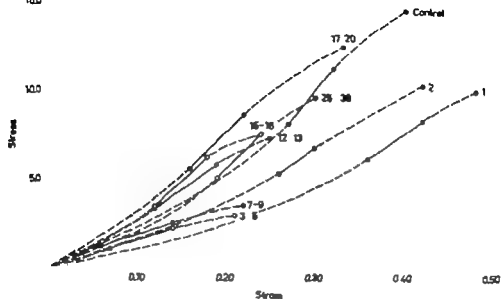


Fig 12 Mean stress-strain curves of uterine horn specimens of young rats during the post partum period. The figures at the tops of the curves denote days after parturition.

maintained until about 12-13 days at which time an increase occurs and continues until the late post partum period. During the early phase the deformation of the specimens is pronounced. During the first day the elongation at the breaking point is 7.5 mm, for the second day 4.4 mm and at 15-16 days this value is only 0.6 mm. The "elastic stiffness" is low during the early post partum period but increases after the 12th day towards the control value, but this is not reached during the time period studied.

The stress-strain relationships are shown in Figs. 11 and 12 Table A II. During gestation the strain is reduced for the coordinates measured. The most pronounced reduction occurs in the 21 day group where the maximum strain is 0.15 compared to the control value of 0.40. The stress is greatest for the control rats and decreases somewhat but not as obviously as for the strain values. For the maximum stress the decrease is least evident, the value for the 19-20 day group being in the range of the control group. With a small reduction in the stress values and a large reduction for the strain, the

Table II Wet weights of the uterine horns (mean values \pm S.E.M.) from old rats.

No of pregnancies	No of animals	\bar{x}
0=control	9	0.910 ± 0.100
4	14	0.740 ± 0.060
5	10	0.720 ± 0.070
6	4	0.830 ± 0.180
4+5+6	28	0.751 ± 0.044

modulus of elasticity* tends to increase near parturition, although the differences are not significant compared to the control group. From the appearance of the curves the post partum period can be divided into different phases. The first and second day groups show increases in maximum strain and decreases in maximum stress compared to the control, but compared to the late gestational groups the stress is not altered but the strain is. From the 3rd to the 9th day when the collagenous framework is broken down, stress and strain values are very low indicating a loss of the force resisting properties. From the 12th day and onward stress values increase and the 17-20 day group almost reaches the control group values. During the latter half of the post partum period there is thus a restoration of the biomechanical properties. This phenomenon is also seen in the values for the elastic stiffness.

Old multiparous rats The fresh weights of the horns (Table II) in the multiparous groups all tend to have lower weights compared to the virgins although the differences are not significant ($2P < 0.05$)

The amount of collagen per unit specimen length (Table III) for the

Table III Amount of collagen per unit specimen length of uterine horn specimens (mean values \pm S.E.M.) from old rats.

N of pregnancies	N of animals	mg/mm
0=control	9	0.2547 ± 0.0266
4	14	0.1508 ± 0.0115
5	10	$0.1107 \pm 0.0092^*$
6	4	$0.1755 \pm 0.0228^{**}$
4+5+6	28	$0.1400 \pm 0.0084^{**}$

$P < 0.05$ against control.

Table IV The original length (l_0) of uterine horn specimens (mean values \pm S.E.M.) from old rats

No of pregnancies	No of animals	
0=control	9	5.2 ± 0.2
4	14	5.0 ± 0.3
5	10	5.5 ± 0.5
6	4	5.0 ± 0.8
4+5+6	28	5.2 ± 0.5

multiparous animals in the different groups is significantly higher than that of the control group. The original lengths of the specimens (Table IV) do not differ very much between the groups. The pooled group of all multiparous animals has actually the same mean value as the control group.

The biomechanical properties of the uterine horn specimens given in

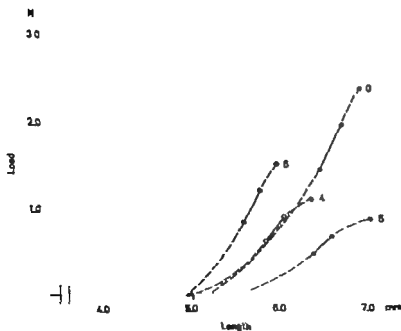


Fig 13 Mean load-length curves of uterine horn specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

nominal values are presented in Fig 13 and Table A I. The deformation values for the multiparous animals compared to the virginal ones does not differ significantly in any of the coordinates measured. The lowest values are found in the 4 and "6" pregnancies groups. Except for the "6" pregnancies group the load is significantly lower for the coordinates measured. This is in accordance with the amount of collagen per unit specimen length which was highest in the control group. The elastic stiffness" parallels the load readings with low values among the multiparous animals. The stress-strain relationship, which is presented in Fig 14 and Table A II shows that the strain is significantly reduced at the start and end of the linear region for the multiparous animals. For the maximum strain only the 6" pregnancies group is significantly reduced compared to the virgins. The stress values for the control group tend to be higher than for the multiparous animals but the differences are not significant. Also the values for the modulus of elasticity" show only small variations.

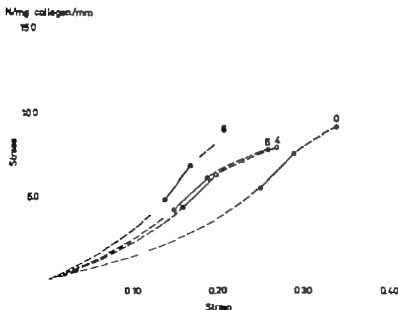


Fig 14 Mean stress-strain curves of uterine horn specimens of old multiparous rats. The figures at the top of the curves denote number of pregnancies.

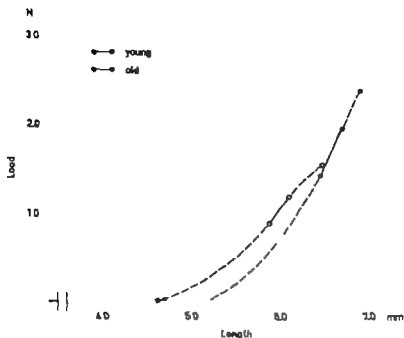


Fig. 15 Mean load-length curves of uterine horn specimens of young and old virgin rats.

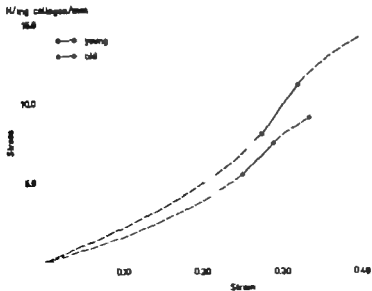


Fig. 16 Mean stress-strain curves of uterine horn specimens of young and old virgin rats.

Young and old virgin rats The biomechanical properties for these animals are compared in Figs. 15 and 16 and Table A I and A II. The load-deformation relationship shows only slight differences with higher values for the old animals. Compared to the young rats the original length has increased from 4.6 to 5.2 mm in the old ones. The amount of collagen per mm specimen length shows a significant increase of more than 100 per cent in the old animals. These figures explain the higher load-deformation values for the old animals.

The stress-strain relationship however shows higher values for the young animals, and the stress values for the end of the linear region and for the breaking point are significantly different from the old rats. Also the modulus of elasticity is decreased in the old virgins.

Uterine cervix

Young pregnant rats The wet weight of the cervix (Fig. 17) increases approximately 100 per cent during gestation and decreases after parturition reaching about 50 per cent below the value of the non pregnant animals. During the late post-partum period the weight increases again and is in the range of the control animals. The amount of collagen per unit length of the specimen, shown in Fig. 18 decreases during gestation with about 50 per cent. The values during the post partum phase vary to some extent. In the early post partum period the values are much greater than those found in late gestation and also greater than for the non pregnant animals. Then the value tends to decrease and in the late post partum period it is similar to that found in the non pregnant rat.

The original length (Fig. 19) starts to increase at approximately the 13-14 day of gestation, and reaches its highest value just before parturition. During the involution period the cervical diameter decreases noticeably the first day after parturition and then somewhat more slowly. During the late post partum phase the values reaches those of the control animals.

The load length values are given in Figs. 20-21 and 22 and those for load-deformation in Table A III. During gestation the start of the curve moves along the length axis due to the increase in the circumference of the original length. There is also an increase in the deformation of the curve. The maximum deformation at the rupture point is 1.9 mm in non-pregnant animals

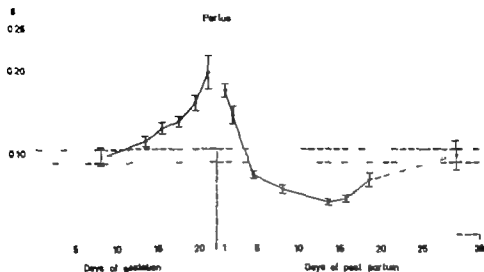


Fig 17 Wet weights of the uterine cervix of young rats during pregnancy (Mean values \pm S.E.M.)

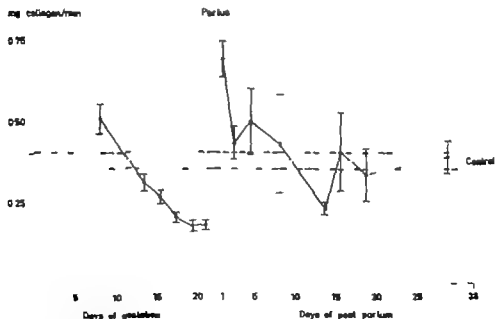


Fig 18 Amount of collagen per unit specimen length of uterine cervix specimens of young rats during pregnancy (Mean values \pm S.E.M.)

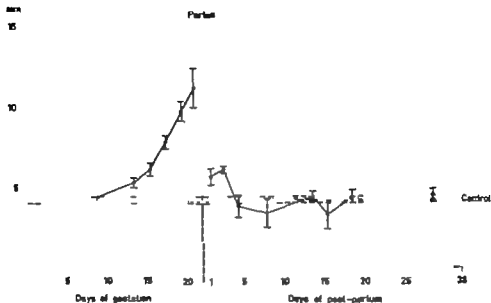


Fig 19 Original length (l_0) of uterine cervix specimens of young rats during pregnancy (Mean values \pm S.E.M.)

M
15

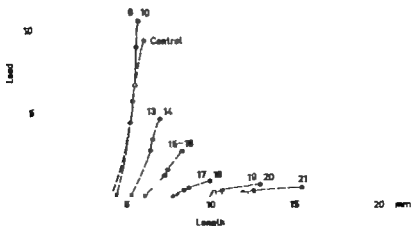


Fig 20 Mean load-length curves of uterine cervix specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

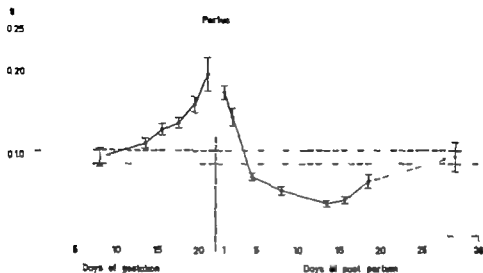


Fig 17 Wet weight of the uterine cervix of young rats during pregnancy (Mean values \pm S.E.M.)

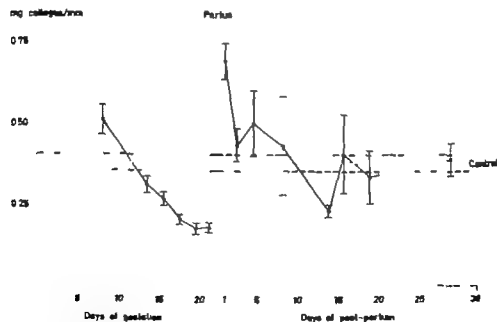


Fig 18 Amount of collagen per unit specimen length of uterine cervix specimens of young rats during pregnancy (Mean values \pm S.E.M.)

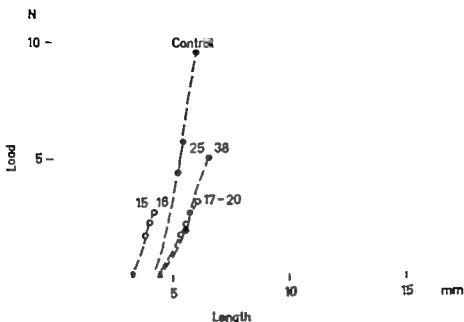


Fig. 22 Mean load-length curves of uterine cervix specimens of young rats during the late post-partum period. The figures at the tops of the curves denote days after parturition.

significant increase on the first day compared to late gestation. Then there is a pronounced decrease in this value and although the values again rise in the late post partum phase they do not reach the virginal level within the period of observation. The "elastic stiffness" follows the variation in the load values as compared to late gestation. The value increases on the first day but then decreases when the collagen is broken down. During the late post partum period the "elastic stiffness" again increases.

The stress-strain relationships are presented in Figs. 23, 24 and 25 and Table A IV. During gestation there is a decrease of the strain values most pronounced during late gestation. The stress values decrease during gestation with about 90 per cent for all the coordinates measured. At the time of parturition the force resisting properties of the collagenous framework is reduced. This is, however, not the case for the cervixes in the group at one day post-partum. The mean curve for this group has a much more pronounced toe

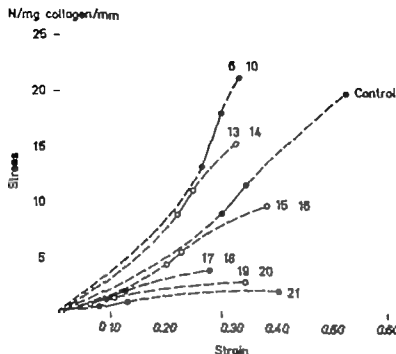


Fig 23 Mean stress-strain curves of uterine cervix specimens of young rats during pregnancy. The figures at the tops of the curves denote days after gestation.

or less resemble those of the control group, although the stress remains somewhat lower. The "modulus of elasticity" changes in parallel with the stress values with an increase for the last group.

Old multiparous rats. In Table V the wet weights of the whole cervixes are given. The differences between the multiparous groups are slight, but in comparison to the virgin animals there is a tendency for an increase, which, however is not significant ($2P < 0.05$).

In Table VI the amount of collagen per unit specimen length and in Table VII the original length (l_0) are given. The circumference of the uterine specimens is not influenced by repeated pregnancies, as the values for the virgin group and for all multiparous rats are almost the same. The amount of collagen tends to increase for the "6" pregnancies group while for the other groups no differences are found when compared to the virgins.

In Fig 26 and Table A III load-deformation values for the multiparous

N/mg collagen/mm

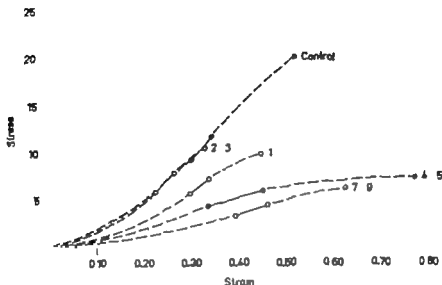


Fig 24 Mean stress-strain curves of uterine cervix specimens of young rats during the early post partum period. The figures at the tops of the curves denote days after parturition

part and higher stress values compared to late gestation. During the period of collagen catabolism there is a larger strain at low stress and the toe-parts are fairly long. During the later part of the post partum period the curves more rats are presented. The mean values for both the multiparous and the pooled virginal animals are fairly similar and there are no significant differences for any of the parameters recorded.

The stress-strain relationships are presented in Fig. 27 and Table IV. Only for the end of the linear region in the 4th pregnancies group are the strain values significantly increased compared to the virginal animals, while the strain as well as stress values for the other parameters show no significant differences.

Young and old virginal rats. The biomechanical properties are compared for these animals in Figs. 8 and 29 and Table A III and A IV. In the old animals the original length is increased 60 per cent and the amount of collagen per unit length approximately 100 per cent. The wet weight is also increased

N/mg collagen/mm

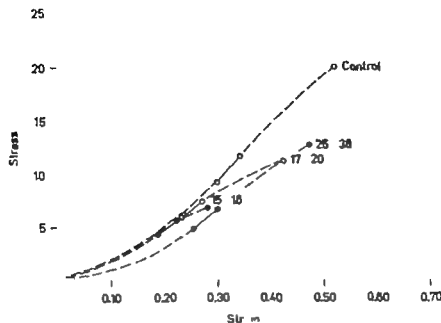


Fig 25 Mean stress-strain curves of uterine cervix specimens of young rats during the late post-partum period. The figures at the tops of the curves denote days after parturition

Table V *Uterine weights of the uterine cervix (mean values \pm S.E.M.) from old rats.*

No of pregnancies	N of animals	\bar{x}
0=control	6	0.170 ± 0.020
4	10	0.210 ± 0.010
5	9	0.190 ± 0.010
6	4	0.210 ± 0.020
4+5+6	23	0.205 ± 0.008

$2P < 0.10$ against control.

about 10 per cent. Although there is significantly more collagen in the specimens from the old animals, the load values compared to the young ones are only slightly but not significantly increased. Due to the increase of the original

Table VI Amount of collagen per unit specimen length of uterine cervix specimens (mean values \pm S.E.M.) from old rats.

No of pregnancies	No of animals	mg/mm
0=control	6	0.8100 ± 0.1119
4	10	1.0118 ± 0.1134
5	9	0.8800 ± 0.0966
6	4	1.0397 ± 0.0504
4+5+6	23	0.9651 ± 0.0870

$2P < 0.10$ against control.

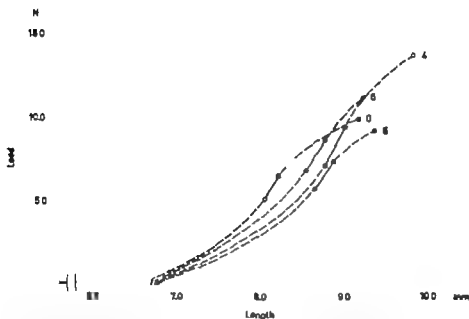


Fig. 26 Mean load-length curves of uterine cervix specimens of old multiparous rats. The figures at the top of the curves denote number of pregnancies.

length the deformation values of the specimens in the old group is significantly greater. The "elastic stiffness" is the same in both groups.

Both strain and the "modulus of elasticity" values are the same in young and old rats, but the stress values are significantly lower in the old group.

Table VII Original length (l_0) of uterine cervix specimens (mean values \pm S.E.M.) from old rats.

N of pregnancies	No of animals	mm
0=control	11	6.6 ± 0.3
4	10	6.7 ± 0.2
5	9	6.8 ± 0.3
6	4	6.7 ± 0.6
4+5+6	23	6.7 ± 0.2

Vaginal wall

Young pregnant rats The wet weight of the vagina (Fig. 30) is increased during the first half of the gestation and the maximum value is reached just before parturition. As with other parts of the genital tract, there is a weight reduction during the first half of the post partum phase, which, however is not so pronounced compared to the uterine horns and cervix. A

M/mg collagen/mcm

75

10

Stress

5

0.10

0.20

0.30

0.40

0.50

Strain

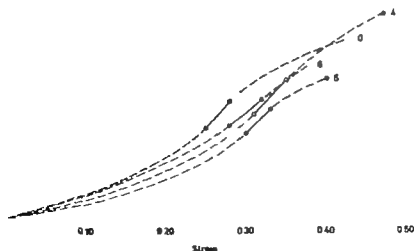


Fig. 7 Mean stress-strain curves of uterine cervix specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

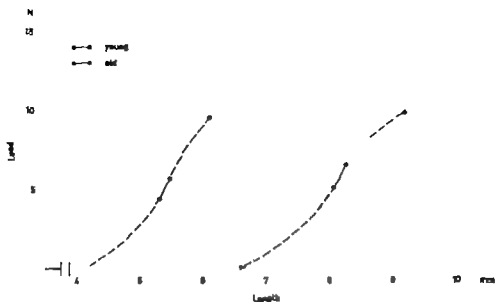


Fig 28 Mean load-length curves of terine cervix specimens of young and old virginial rats.

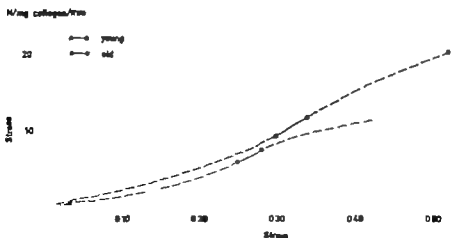


Fig 29 Mean stress-strain curves of terine cervix specimens of young and old virginial rats.

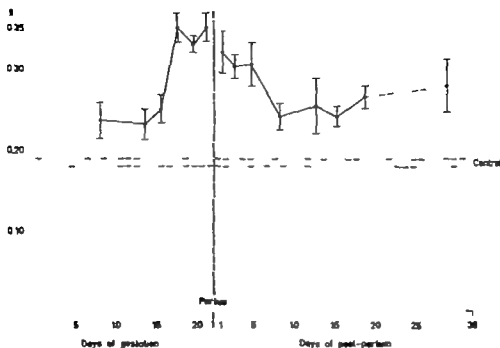


Fig 30 Wet weight of the vagina of young rats during pregnancy (Mean values \pm S.E.M.)

weight increase seems to persist at the end of the post-partum period studied in comparison to the non-pregnant animals. The amount of collagen per unit length (Fig 31) decreases during gestation by a little less than 50 per cent towards parturition. During the involution period higher values than at the end of gestation are noted for the first week followed by a decrease, and in the late post partum the values approach those of the non pregnant state.

The variation of the original length during pregnancy is shown in Fig 32. The increase during gestation is notable in the 6-10 day group and the maximum value is reached around parturition with a 60 per cent increase compared to the controls. After parturition the original length decreases and during the late post partum period approaches the values of non pregnant rats.

The load length relationships for the vaginal wall during the gestation and post partum periods are shown in Figs. 33 and 34 and the load-deformation

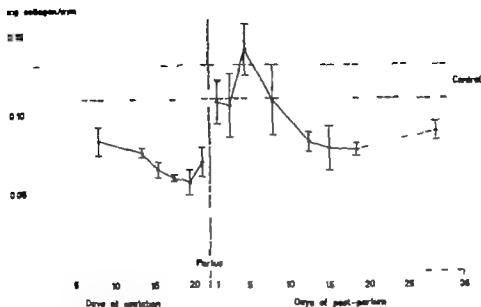


Fig. 31 The amount of collagen per unit specimen length of vaginal wall specimens of young rats during pregnancy (Mean values \pm S.E.M.)

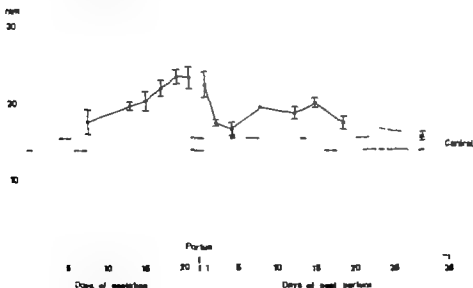


Fig. 32 Original length (l_0) of vaginal wall specimens of young rats during pregnancy (Mean values \pm S.E.M.)



Fig. 33 Mean load-length curves of vaginal wall specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.



Fig. 34 Mean load-length curves of vaginal wall specimens of young rats during the post-partum period. The figures at the tops of the curves denote days after parturition.

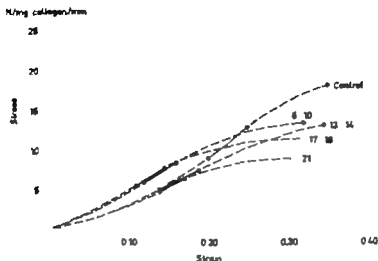


Fig. 35 Mean stress-strain curves of vaginal wall specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

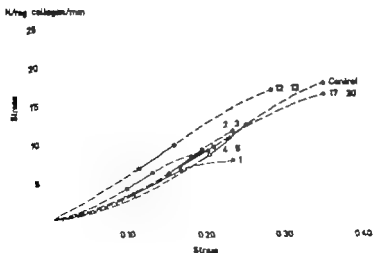


Fig. 36 Mean stress-strain curves of vaginal wall specimens of young rats during the post-partum period. The figures at the tops of the curves denote days after parturition.

parameters in Table A V. The deformation of the specimens at failure increases during gestation. In non-pregnant animals this value is 4.9 mm, in the 15-16 day group 6.0 mm and at the 21st day 7.1 mm. The breaking load decreases more than 3 times to the time of parturition. This reduction is most pronounced in the first half of gestation. There is a decrease in elastic stiffness from 0.7 N/mm in non-pregnant animals to 0.2 N/mm just before parturition. During the involution period the maximum deformation is slightly reduced and the maximum load is increased. The "elastic stiffness" parallels the changes in the load readings showing increasing values during the post partum phase. During the late post partum period the values are close to the non-pregnant level.

The corresponding stress-strain values are presented in Figs. 35 and 36 and Table A VI. Throughout gestation the strain is with a few exceptions lower for the start and end of the linear region. The toe-part is less pronounced in gestational than in non-pregnant animals. The stress values are, with the exception of the 6-10 and 19-20 day group lower during gestation compared to the control. The ultimate stress at the 21st day is less than half the value for the non-pregnant animals. The "modulus of elasticity" is, however only decreased during gestation for the 13-14 day group.

The stress-strain curve at one day post partum is very similar to the curve for the 21st day of gestation. The strain values remain generally significantly lower during the post partum period than the controls but increase in the late phase of involution. The stress values are also lower but beginning from about one week they gradually increase and reach the non-pregnant level about the 14-15th day. The "modulus of elasticity" is significantly decreased

Table VIII IV : weights of the vagina (mean values \pm S.E.M.) from old rats.

N of pregnancies	N of animals	g
0=control	9	0.297 \pm 0.033
4	15	0.387 \pm 0.013**
5	9	0.402 \pm 0.029**
6	4	0.383 \pm 0.049
4+5+6	28	0.392 \pm 0.013**

** 2P<0.05 against control.

Table IX Amount of collagen per unit specimen length of vaginal wall specimens (mean values \pm S.E.M.) from old rats.

No of pregnancies	N of animals	mg/mm
0=Control	9	0.1757 \pm 0.0142
4	15	0.1841 \pm 0.0127
5	9	0.1677 \pm 0.0153
6	4	0.1056 \pm 0.0103
4+5+6	28	0.1664 \pm 0.0093

$P < 0.05$ against control.

one day after parturition, but otherwise during the post partum period the values are in the range of the controls.

Old multiparous rats The wet weight of the vagina (Table VIII) is significantly increased in the multiparous animals by about one-third compared to the virgins. For the pooled group of multiparous animals there is no significant difference in the amount of collagen per unit specimen length (Table IX) compared to the vaginal rats but the value of the "6 pregnancies group" is significantly lower in comparison to the other two multiparous groups and the control group. The vaginal group has the shortest original length (Table X) and is significantly lower compared to the 6 pregnancies group.

The load length relationships for the vaginal wall are presented in Fig. 37 and the load-deformation parameters in Table A V. Since the original length

Table X Original length (l_0) of vaginal wall specimens (mean values \pm S.E.M.) from old rats.

N of pregnancies	No of animals	mm
0=control	9	14.6 \pm 1.0
4	15	15.6 \pm 0.8
5	9	17.2 \pm 1.4
6	4	20.5 \pm 2.5
4+5+6	28	16.8 \pm 0.7

$2P < 0.10$ against control

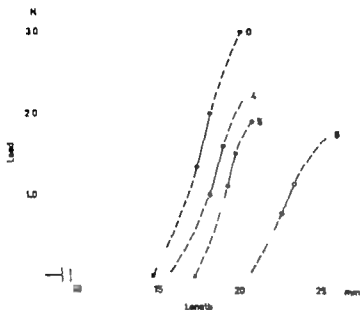


Fig 37 Mean load-length curves of vaginal wall specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

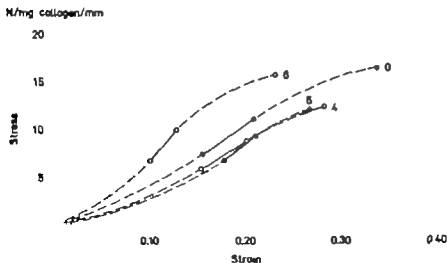


Fig 38 Mean stress-strain curves of vaginal wall specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

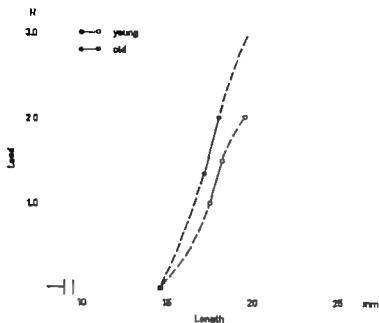


Fig 39 Mean load-length curves of vaginal wall specimens of young and old liginal rats.

increases with the number of pregnancies, the curves for the respective groups move along the length axis. The total deformation does not vary to any noticeable degree between the groups, being at most 5.3 mm for the virginal group and at least 3.3 mm in the 5th pregnancies group. The differences between virgins and multiparous animals are more pronounced for the load values where the breaking load is significantly lower for the multiparous groups. The "elastic stiffness" tends also to be lower for the multiparous animals although not significantly different compared to the control group.

The stress-strain values are presented in Fig 38 and Table A VI. The strain values are lower for the "6" pregnancies group than for the other groups. The differences are, however slight when comparing the pooled multiparous animals with the virginal ones. The maximum stress values are significantly reduced in the "4" and 5 pregnancies groups compared to the virgins, but for the other stress parameters no differences are found. The modulus of elasticity is in the same range for all the groups.

N/mg collagen/mm

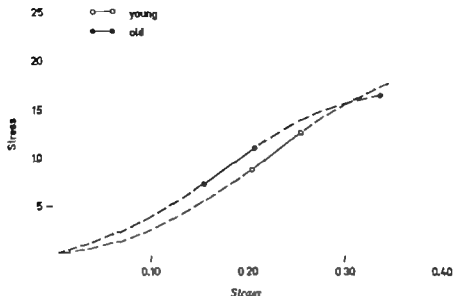


Fig 40 Mean stress-strain curves of vaginal wall specimens of young and old virgin rats.

Young and old virgin rats The load length curves for these animals are compared in Fig 39 and the load-deformation values in Table A V. The deformation values are approximately the same in the two groups reaching about 5 mm at the rupture point. The load value for the old rats is significantly higher at the breaking point. There is no difference between the "elastic stiffness" values.

The stress-strain relationships are presented in Fig. 40 and Table A VI. The differences between the groups are smaller than for the load-deformation values. Both the stress and strain values are in the same range for both groups.

Discussion

Uterine horns

Young pregnant rats The increase of the wet weight of uterine horns during

gestation (Fig. 6) is approximately eight times that found in virgin rats. This is slightly more than was reported by Harkness and Harkness (1954). The decrease of the wet weight during the post partum period is in agreement with other investigations (Harkness and Harkness, 1954; Maibenco 1960). As there is a slower increase in the total amount of collagen than in the wet weight, the collagen concentration decreases (Harkness and Harkness, 1954). In the present experiment the concentration expressed as the amount of collagen per unit specimen length, decreased 4.5 times during gestation (Fig. 7). During the early post partum period the reduction of the wet weight is more rapid than that of the collagen (Harkness and Harkness, 1954) which results in that the amount of collagen per unit specimen length increases compared to the end of gestation although no new collagen is probably formed.

Reports on the decrease of collagen concentration during gestation in the uterine horns of the rat vary somewhat. Harkness and Harkness (1954) found a reduction from 2.6 to 2.2 per cent of wet weight. Monfort and Perez Tamayo (1961) on the other hand also found on wet weight basis a 50 per cent reduction during gestation. In both these investigations the control rats were in the estrus phase. In the present study where the amount of collagen per unit specimen length decreased 4.5 times the control animals were in diestrus phase. The wet weight of the uterine horns is approximately one third lower in diestrus than in estrus but the collagen content is not changed (Harkness et al. 1956). Thus the collagen concentration during the estrus cycle will vary with about 50 per cent. This can partly explain the differences discussed above.

The load-deformation and load-length relationships show the progressive changes of the physical properties of the horns during the pregnancy. A reduction of the force resisting properties is very evident at the end of gestation when the breaking load is reduced 5-6 times compared to the non pregnant animals. This parallels the decrease in the amount of collagen per unit specimen length which is reduced more than 5 times during gestation. The growth of the uterine horns during gestation results in an increased circumference (Fig. 9). The growth of the wall in the horns is not as rapid as the contents, i.e. the fetuses and placentae. The wall becomes thinner and the amount of collagen per unit area (mg/cm^2) is reduced 5-6 times during gestation (Harkness and Harkness, 1956). From the photographs on which the original lengths were determined, a progressively thinning of the wall during gestation could

tion of the cervix has been a combination of inspection and palpation, which seems satisfactorily reproducible. The increase in wet weight during gestation according to the above mentioned authors is somewhat more than 200 per cent from diestrus values, but in this investigation it is only a little more than 100 per cent. The decrease of the wet weight after parturition is not as pronounced during the first and second day as reported by Zarrow and Yochim (1961) but thereafter the reduction is in agreement with other authors.

Harkness and Harkness (1959a) found that the inner circumference during gestation increased almost 400 per cent compared to non-pregnant animals. In the current investigation the original length increases almost 300 per cent. The collagen concentration during gestation decreased 40 per cent according to Harkness and Harkness (1959a) and according to Zarrow and Yochim (1961) 60 per cent. In the current report the amount of collagen per unit specimen length decreases a little more than 50 per cent. During the post partum phase the collagen concentration increases the first few days because of a more pronounced reduction of other tissues (Harkness and Harkness 1956 Zarrow and Yochim, 1961) (Cf. Fig. 18)

These changes indicate that an extensive morphologic rearrangement takes place in the uterine cervix during pregnancy. The physical properties are profoundly influenced by these changes. Harkness and Harkness (1959a) found the maximum load for the whole uterine cervix of non pregnant rats to be approximately 900 g, 975 g on the 11-12th day of gestation and about 300 g on the 21st day. In the present series the decrease of breaking load is much greater more than 20 times. There is of course a difference in the specimens. Harkness and Harkness (1959a) used whole cervixes but in the present study 2 mm wide "ring" specimens are used. This might be of lesser importance when the collagenous framework is more compact as in non pregnant animals but at the end of gestation increased amount of other tissue components such as ground substance (Cf. Bryant et al. 1968) separates the collagen fibers. Cutting of fibers in the delicate cervical reticulum might disturb the integrity of the collagenous framework and result in lower values for the load readings. During gestation the cervical specimens show an increase in deformation and a decrease in load. At the end of gestation the total deformation at rupture is more than 5 mm at the load of 0.4 N compared to the non pregnant group which has a deformation of 1.9 mm at a load of 9.5 N. These changes are also reflected in the more than 40 times decrease in the

"elastic stiffness" values. These changes recorded for the cervical specimens are in accordance with physiological events at parturition. The low loads required for the dilation of the uterine cervix prevent harming of the fetuses. The breaking load according to Harkness and Harkness (1961) was one day post partum 900 g. This is followed by a decrease to approximately 300 g at the 8th day and then at the 16th day a slight increase is noted indicating a restoration of the collagenous framework. These values are similar to those found in the present investigation. During the post partum period the maximum load as well as the other load parameters never reach the low values recorded prior to parturition.

During gestation the decrease in the strain values is most pronounced at the start and end of the linear region of the curves resulting in a diminished toe part of the curve. This can be due to a stretching of the collagenous framework that increases the circumference since other tissue compartments increase more than the collagen content (Harkness and Harkness 1959a). The maximum strain values do not show such a great decrease during gestation. This might be due to an increased amount of ground substance (Bryant et al. 1968) and maybe other changes that allow the fibers to yield or slide in response to mechanical stress above the linear part of the curve.

The stress values during gestation also decrease. The ultimate stress value at the 21st day is 10 times lower compared to the non-pregnant value. There must be qualitative changes or changes in the structural organization in the force resisting properties of the collagenous framework. Harkness and Harkness (1959a) reported that the tension at break in N/mm² collagen for non-pregnant diestrus rats is 19 and for the 21st day of gestation, 10. Although the differences in the present investigation are more pronounced partly due to the shape of the specimens, the results are in line with those of Harkness and Harkness (1959a).

Harkness and Harkness (1961) reported that a slight increase of the ultimate tensile strength was noted one day after parturition but Zarrow and Yochlin (1961) found this to be 4 times compared to the value at parturition. Such a change is also found in the present investigation. Also the amount of collagen per unit specimen length was remarkably increased one day after parturition but the total amount of collagen was only slightly reduced at this time (Harkness and Harkness, 1961). Bryant et al. (1968) reported that the concentration of hexosamine was 2.51 at parturition and one day later 1.24 g per 100 g

freeze-dried cervical tissue. Thus it might be assumed that there is a condensation of collagen and due to the closer packing a regain of some of its force resisting properties. According to Harkness and Harkness (1961) the "extensibility" which was most pronounced at parturition was completely lost one day later. This indicates that the cohesive forces between the collagen units is now stronger than at the time of parturition. During the days following parturition the stress values decrease when the process of collagen break down is most active. Later there is again an increase, a reconstitution of the collagen framework and the stress-strain curves look more like the ones for the controls. The change in the strain values one week post partum results in pronounced toe parts of the stress-strain curves. At this time the collagen catabolism is most active.

Old multiparous rats During the post partum period there is a reconstitution of the uterine cervix towards the non-pregnant state, but the question remains whether repeated pregnancies will permanently change the properties of both the cervix as a whole and its force resisting material per se.

The wet weight of uterine cervix in multiparous animals tends to be somewhat higher compared to virginal animals. This is contrary to what was found in the uterine horns where the controls have the highest value. Harkness and Moralee (1956) showed that the wet weights of both the uterine horns and uterine cervixes were reduced almost seven times during the first week of the involution period, reaching values below the non-pregnant level. The original length of the cervical specimens shows hardly any differences while the amount of collagen per millimeter specimen length in the control group has the lowest value. The tendency for the increase of collagen in the multiparous rats is not significant compared with the controls. This is again contrary to the findings in the uterine horns where the virgins have a significantly increased amount of collagen compared to the multiparous animals. The loss of collagen from the uterine horns after parturition was, however more pronounced than in the uterine cervix (Harkness and Moralee, 1956). With repeated pregnancies this could result in an increasing amount of collagen in the cervix compared to the horns.

The load-deformation and stress-strain curves are similar between multiparous and virginal animals. The force resisting properties of the collagenous framework thus seem to be almost unaffected by repeated pregnancies.

Young and old virginal rats The load-deformation curves are almost the

same in both groups, although the amount of collagen per unit specimen length and original length are increased in the old animals. The stress values for the old rats are significantly decreased. Although the amount of collagen increases the force resisting properties of the collagen per se in the uterine cervix deteriorates by age. This process is not influenced by multiparity.

Vaginal wall

Young pregnant rats. The changes in the vaginal wall during pregnancy are similar to those in other parts of the reproductive tract although they are less pronounced. The 100 per cent increase in fresh weight during gestation is in good agreement with the findings of Harkness and Harkness (1965). After parturition the weight reduction is less pronounced without hyperinvolution as in the uterine horns and cervix, and the non pregnant values are not reached even in the late post partum period. The original length increases about 60 per cent during gestation and at the 21st day the value of the circumference approximated to twice the original length, is 46 mm. This value is in agreement with Harkness and Harkness (1965). However at one day after parturition these authors found for this value a more than 30 per cent decrease compared to late gestation. This is not found in the present investigation. The reduction in the original length at this time is very slight but by the 4th-5th day post partum the reduction is about one-third compared to late gestation.

The amount of collagen per unit specimen length decreases about 50 per cent during gestation compared to diestrus controls. Harkness and Harkness (1965) found the collagen concentration to decrease during the same period about 40 per cent compared to non pregnant rats in diestrus. The increase of collagen during gestation does not keep pace with the total increase in wet weight. Thus other components in the vaginal wall must increase more (i.e. the amount of the ground substance) as was shown histologically in pregnant women (Rachm, 1951). Such changes result in a looser collagenous fiber system. The deformation of the specimen at rupture is increased 1.5 times at the end of the gestation and the load values are reduced 3-4 times. These changes might be a physiological anticipation of parturition allowing a dilatation of the vaginal wall at fairly low forces. After

parturition the deformation decreases somewhat and the load increases without being reduced during the first and second week as in the uterine cervix and horns.

In the stress-strain relationships the strain values are only slightly affected during gestation. The earlier start of the linear region can be due to the adaptability of the collagenous framework to the increased circumference. Since collagen is laid down to a lesser degree than other tissue components, the fibers probably will be less relaxed than in the non pregnant animals. The stress values are reduced during gestation although not so pronounced as for the uterine cervix. The maximum stress at the 21st day is thus about one half the value in the non pregnant rats.

After parturition the stress-strain values remain low for about one week but then there is an increase towards the non-pregnant state. The changes during this period are less pronounced in the vaginal wall than in other parts of the genital tract. The curve for the 17-20 day group is very close to the curve for the control group. The force resisting properties of the collagenous framework of the vaginal wall is reduced during pregnancy. It is possible that this reduction might be caused by an increased amount of ground substance that separates the collagenous fibers resulting in a lowered aggregation and a lowered ability to withstand forces. This mechanism might also be responsible for the increase in "extensibility" at the end of gestation that was found by Harkness and Harkness (1963). This "extensibility" is more pronounced on the 12th than 21st day of gestation and returns to non-pregnant values 24 hours after parturition. In the data of the present investigation there is no explanation for the fact that the "extensibility" is greater during mid term than at the time of parturition.

Old multiparous rats After a single pregnancy the reduction of the wet weight during the post-partum period studied does not reach the non-pregnant level. It might thus be assumed that a weight gain will occur with repeated pregnancies which is also the case. All multiparous rats together show no difference with regard to the amount of collagen per unit specimen length compared to the virgins. The weight gain is thus caused by other tissue components than collagen.

The physical properties are also influenced by repeated pregnancies as the maximum load is significantly decreased in the multiparous animals compared to the virgins. In the stress-strain relationships the maximum stress is

significantly lower for the multiparous animals compared to the virgins. The collagenous framework of the vaginal wall thus loses some of its force resisting properties due to repeated pregnancies.

Young and old virgin rats In the old animals the wet weight and the amount of collagen per unit specimen length increase. An increase of collagen in the vaginal wall due to aging was also reported in a histologic investigation of mice by Loeb et al. (1939). In old rats the maximum load value is increased due to the increased amount of collagen but the stress-strain curves are similar between the two groups. The functional properties of the collagen per se as expressed in its force resisting ability are not influenced by aging. Repeated pregnancies seem thus to exert a greater influence on the physical properties, as discussed above, than aging.

Part II

BIOMECHANICAL PROPERTIES OF EXTRAGENITAL COLLAGENOUS STRUCTURES

Introduction

Morphologic and functional alternations of the collagenous framework of the reproductive tract as influenced by a single pregnancy repeated pregnancies and aging have been studied fairly extensively (Cf Part I) Harkness (1964) considers it unlikely that the underlying mechanisms are active only locally but rather represent examples of processes of more general character

Relatively little attention has, however been paid to these processes outside the genital tract, with the exception of the pubic symphysis, which at least in some species can be considered a semigenital target organ. The extragenital connective tissues during pregnancy and after repeated pregnancies have been studied only by measuring the isotonic thermal reactivity of rat tail tendons (Cf. Part III)

Pubic symphysis

Young pregnant rats There are some reports in the literature concerning changes of the pubic symphysis during pregnancy (Hisaw and Zarrow 1950 Frieden and Hisaw 1953 Storey 1957 Grelin and Brightman, 1957) As these changes are most pronounced in mice and guinea pigs, the reports mainly deal with these species, where alterations during pregnancy involve a complete change of the structure of the tissue.

The cartilagenous symphysis in the non-pregnant mice is replaced by a loose ligament during pregnancy and the distance between the pubic bones increases five times (Storey 1957) The collagen bundles swell and stain more easily with periodic acid-Schiff reagent and silver impregnation methods. Comparison between load-deformation curves for the pubic symphysis from a

non-pregnant and a gestational mouse at the 19th day showed greatly reduced load and increased deformation values for the latter. After parturition collagen bundles come closer to each other. The ligament is reduced in length and the pubic bones come together. There is an increased flexibility in the pubic symphysis of the rat during pregnancy although histologic investigations showed no morphological changes in the joint during this period. Histologically the pubic symphysis in the rat has an intricately arranged three-dimensional collagen fiber net work (Crelin and Brightman 1957).

Old multiparous rats No information concerning changes with aging of the physical properties in the pubic symphysis is available, nor are there any reports concerning the influence of repeated pregnancies. In mice and guinea-pigs the fibrous cartilage is restored after pregnancy and it is not known whether this chronologically younger cartilage also is biologically younger.

Posterior cruciate ligament

Knee joint ligaments have been used by several investigators as convenient test specimens. These structures consist of welldefined fairly parallel-fibered collagenous tissue with insertions into the tibia and the femur. The bones or parts of them can easily be fastened without risking slipping or jaw breaks into clamps constructed for material testing machines. Some investigators used the anterior cruciate ligament (Smith, 1954; Vildik et al. 1965; Vildik 1966, 1968c) while others have used the collateral ligaments (Schild et al. 1967; Tipton et al. 1967, 1969).

It has also been shown that there are no significant differences between right and left legs in measurements of ligamentous strength (Tipton et al. 1967a). Intact ligaments invariably fail at their osseous attachments and variations in breaking force will be those due to changes in the transition ligament nonmineralized fibrocartilage-mineralized fibrocartilage bone (Tipton et al. 1967a). Bone-ligament bone preparations have been used by several investigators for studies on the influence of activity and immobilization on the musculo-skeletal system (Adams, 1966; Vildik, 1968c; Tipton et al. 1967b). The physical properties of ligaments under conditions such as pregnancy and aging have attracted little interest.

Muscle tendons

The mechanical behavior of collagenous tissue as found in muscle tendons has been investigated by numerous authors (for review see Virdik, 1966 1973 Galante, 1967) There is good agreement about the form of the load-deformation or stress-strain curve for this type of tissue When testing isolated pieces of parallel fibered collagenous tissue there has always been problems with the fixing of the tissue in clamps without slipping or producing jaw breaks at too low loads Virdik (1968a) discussed this problem in relation to earlier investigations.

The effect of age changes on the physical properties in muscle tendons have been studied to some extent Rollhäuser (1950) reported tensile strength values of 30 to 45 N/mm² in newborn against 90 N/mm² in adult humans Curtis (1962) reported that old tendons have a higher modulus of elasticity than young ones using a slow rate of straining Changes in stress-strain behaviour of tendons were found during growth but not in senescence (Elden, 1969)

During pregnancy Bryant et al. (1968) measured the amount of hydroxy proline and hexosamine in rat tail tendons from six days before to eight days after parturition. During this period of time no changes in either of these parameters were noted.

Repeated pregnancies in rats have been found to influence tail tendons, the collagen of which differs in its physico-chemical properties from collagen in other organs and species. The aging process in all tendons is a continuous process throughout life and lacks the relatively rapid maturation found in collagen from other tissues during the growth period of the life-span (Piez, 1969) It is unknown whether this reflects a lack of function of these specific tendons in the rat or if there are more profound structural differences on the molecular level compared to other collagenous tissues. It is, however not satisfying to rely only upon experiments from tail tendons, and therefore other non-genital connective tissues have been investigated in the present series of experiments.

Skin

Young pregnant rats Müller (1951) studied the microscopic appearance and

the spreading factor of skin during the 16-20th days of gestation in rats in comparison to virginal controls. The dermis was thicker and contained an abundance of coarse collagenous bundles in the pregnant rats. In addition an increased spread of India ink was found.

Bryant et al. (1968) reported the hydroxyproline concentration to be 5.0 per cent in freeze-dried skin from non pregnant rats. This was reduced to 2.7 per cent in pregnant rats six days before parturition but increased to 5.1 at the time of parturition and to 8.7 four days post partum. On the eighth day after parturition the hydroxyproline concentration had decreased to 5.6 per cent. The total hexosamine content of skin remained essentially unchanged during pregnancy.

The influence of sex hormones on morphological, biochemical and biomechanical parameters of skin has been analysed extensively by Vogel (1970). Small doses of estradiol decreased the maximum load of skin from sexually mature rats during the first and second day of the treatment but larger doses or prolonged treatment with small doses resulted in an increase. Progesterone given in different concentrations increased the maximum load rapidly with only small differences between the groups after the second day.

The collagen content of the skin of rats treated with estradiol is reduced as reported by a number of workers (Lobel et al. 1953, Smith and Allison, 1966, Henneman, 1968) although Morgan (1963) was not able to show this. In hamsters the solubility of skin collagen showed no differences between intact, estradiol treated and untreated, prepubertally ovariectomized animals (Kowalewski, 1969). In young intact female rats given 0.5 mg progesterone daily for 14 days the amounts of salt-soluble and acid-soluble collagen increased and the insoluble fraction decreased compared to untreated controls (Holzman et al. 1964).

The ground substance in skin increases after estrogen stimulus due to increase of hyaluronic acid or chondroitin sulphate (Schmidt, 1958) and these effects are subject to species variations (Warren and Fagan 1960).

Old multiparous rats Vogel et al. (1970) found rat skin from both sexes to increase in ultimate tensile strength reaching a plateau with maturity at about 9 months of age. Thereafter a small decrease occurred with aging. The maximum load did not change much after reaching stable values but the skin thickness increased with the body weight. Fry et al. (1964) reported an increase in breaking strength of approximately three times and a decrease in

"extensibility" (deformation per unit time at constant load) of about 20 times in skin from 20 to 600 days old rats. From the age of three months onwards the collagen concentration did not differ noticeably. Tensile strength increase with age in skin strips was found by Mendoza and Milch (1964/65) when they compared young 70 days of age, and old, 15 months of age, male and female rats. Significant increase was found for both sexes. Significantly higher values were noted in old male compared to old female rats, but no sex differences were found in the young animals.

The possible correlation between the tensile strength of skin strips from rats of different age and the collagen fractions was analyzed by Vogel (1973). The soluble fractions were found to decrease during aging. Insoluble collagen and total collagen per gram of fresh weight showed a maximum at 4 months of age as did the breaking tensile strength.

Materials and methods

Pubic symphysis The specimen consisting of the pubic symphysis and the two coxal bones, when not tested immediately was surrounded with saline-moisten gauze and stored for no more than two hours. The pubic bones were placed into clamps which were contour shaped for the external surfaces of the bones (Fig. 41). The specimen was fixed against the clamp with a polyethylene tube-clad steel rod. The complex was then fastened into the materials testing machine.

Posterior cruciate ligament The knee joint was kept in the hind limb until the testing. All structures except the posterior cruciate ligament were carefully removed from the knee joint. The posterior cruciate ligament was selected because its anatomical position permitted a more rapid dissection than the anterior one. The ligament is in a slightly oblique position, but as the specimens were of fairly uniform appearance the influence of skin on deformation readings was assumed to be constant. The distant end of femur and proximal one of tibia were trimmed in length to fit the contour shaped brass clamps (Fig. 42). The clamps were firmly screwed together and then attached to the materials testing machine.

Muscle tendons The tendons remained in situ until tested. From the lateral muscle group of the hind limbs a muscle tendon was carefully dissected



Fig 41 Contour shaped clamps for the pubic symphysis specimens. During the experiment the specimen is fixed towards the surfaces of the clamps with polyethylene tube clad steel bars.

out. The outermost of the peroneus muscle was cut away. The peroneus digiti quinti muscle which arises from the proximal half of the shaft of the fibula and inserts upon the distal end of the fifth metatarsal bone was freed and its tendon taken for the experiment. The specimen was placed in two saw-toothed clamps with the distance between the edges of the clamps being 3.0 mm when attached to the testing machine. During the experiment the tendon was immersed in a buffered Ringer's solution, pH 7.4 at room temperature.

Skin. The dorsum of the rat was shaved with hair-clippers and a rectangular piece of the middorsal skin was removed. Strips of skin 2 mm wide were cut out using parallel mounted razors. The strips were at a right angle to the cranio-caudal axis. The specimen was then freed from the subcutaneous muscle and mounted in two saw-toothed clamps and mounted in the materials testing machine. During testing the specimen was immersed in buffered Ringer's solution pH 7.4 at room temperature.



Fig 42 Contour shaped clamps for the posterior cruciate ligament specimens. The upper parts of the clamps are in the background and the lower parts with a specimen are seen in the front

Results

Pubic symphysis

Young pregnant rats. The macroscopic appearance of the interpubic joint appears the same all through pregnancy. Microscopically Fig. 43 there seems to be an increase in the ground substance, and the chondrocytes are somewhat more scattered and appear larger and rounded.

The specimens always failed at the junction between one of the bones and the cartilage plate except for a few cases when one of the coxal bones was fastened too firmly in the clamp and accidentally fractured. The specimen was discarded if there were any signs of bone fracture after the test.

The load-deformation relationships are presented in Figs. 44 and 45 and Table A VII. During gestation the deformation values for the start and end of the linear region increase. The maximum deformation, however is only increased in the 21 day group compare to the non pregnant rats. The load



Fig 43 Photomicrographs of pubic symphysis of young rats (formic acid decalcified, hix stained) (a) shows specimen from non-pregnant rat. (b) shows a specimen from one day after parturition. (11 x)

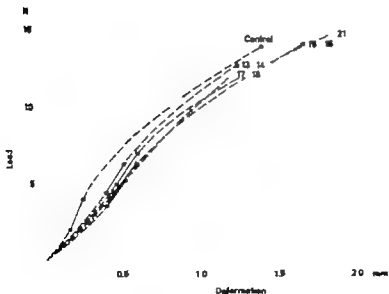


Fig. 44 Mean load-deformation curves of pubic symphysis specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

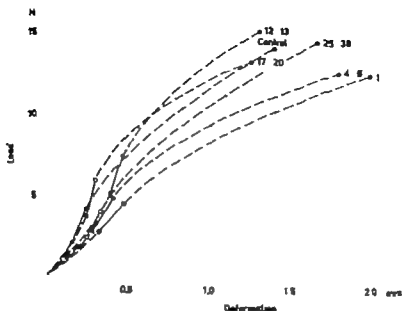


Fig 45 Mean load-deformation curves of pubic symphysis specimens of young rats during the post-partum phase. The figures at the tops of the curves denote day after parturition.

values for the start and end of the linear region is significantly increased in several gestational groups compared to the non-pregnant group. The maximum load is not influenced by gestation being in the same range in all groups as that of the controls. The "elastic stiffness" values are decreased for the 15 16 and 17 18 day groups and tend to decrease in the 19-20 day group compared to the non-pregnant rats. It is noticeable that a few days before parturition the "elastic stiffness" values are greater than during the middle of gestation. The changes in the pubic symphysis during gestation result in larger deformation and lower load values in the first part of the load-deformation curves.

After parturition the deformation values for the start and end of the linear region vary without any clear trend. The values of the 2 3 day and the 12 13 day groups are significantly higher than those of the controls while the values of the other post partum groups are in the range of the non-pregnant animals. The deformation at maximum load remains unchanged also during the post partum period. After parturition the load values for the start and end of the

linear region vary almost in the same way as the deformation values for the same coordinates and the maximum load remains unchanged. One day after parturition the "elastic stiffness" is significantly decreased compared to the non-pregnant group. This is also the case for the 79 day group but during the rest of the involution period, these values for "elastic stiffness" are in the same range as the control group.

Old multiparous rats. As in the young animals the specimens failed at one of the bone cartilage junctions. Due to somewhat thinner and more fragile costal bones in the old animals some of the specimens fractured in the clamps and thereby excluded from the series. Four bone fractures were noted in the non-pregnant group and three in the 4 pregnancies group. The biomechanical properties of the pubic symphysis are presented in Fig. 46 and Table A VII. If the pooled group of all multiparous animals is compared with the virginal group no differences are found for the deformation values. In the 5th pregnancies group the deformation value for the end of the linear region is signifi-

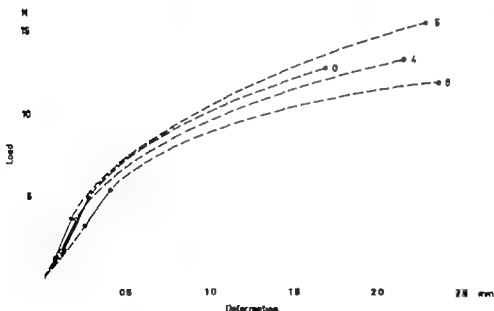


Fig. 46 Mean load-deformation curves of pubic symphysis specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

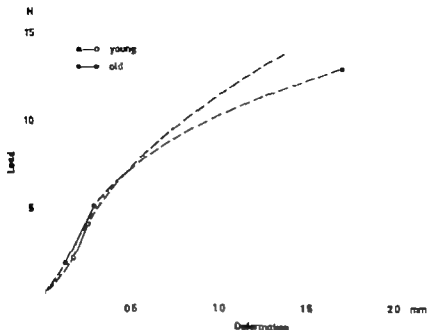


Fig 47 Mean load-deformation curves of pubic symphysis specimens of young and old virginal rats.

cantly lower compared to the virgins. The load value for the end of the linear region is decreased in the multiparous animals while the values for the start of the linear region, maximum load and "elastic stiffness" are not significantly different compared to the control group

Young and old virginal rats The influence of aging on the physical properties of the pubic symphysis (Fig 47 and Table A VII) is very slight. The values for all parameters are in the same range on the two groups except for the load value at the end of the linear region, which tends to increase in the old rats.

Posterior cruciate ligament

Young pregnant rats The specimens invariably failed in one of the bone ligament junctions. The maximum load value will thus express the condition of the bone-ligament junction more than the strength of the ligament itself

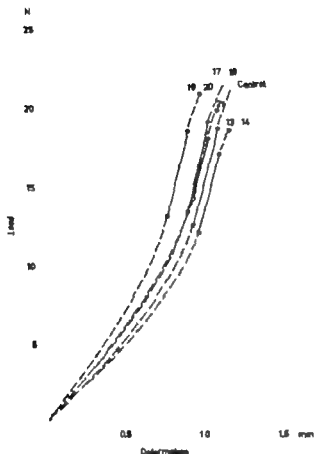


Fig 48 Mean load-deformation curves of posterior cruciate ligament specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

The other parameters, the linear region and the "elastic stiffness" are more relevant for the properties of the collagenous framework in the ligament. The values are presented in Figs. 48 and 49 and Table A VIII

The deformation values during gestation are similar to the non pregnant rats except for two groups. In the 6-10 and 19-20 day groups lower values are recorded. The decreased deformation in the 6-10 day group is followed in the 13-14 day group by values in the range of the control group. During ges-

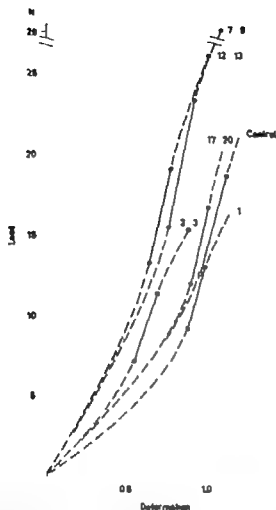


Fig 49 Mean load-deformation curves of posterior cruciate ligament specimens of young rats during the post-partum period. The figures at the tops of the curves denote days after parturition

tation the load values at the start and end of the linear region are in the range of the non pregnant animals. The maximum load values are also in the range of the controls except for the 13-14 day group and this value is significantly decreased. The "elastic stiffness" values are similar to that of the virginal group.

During the first two weeks after parturition the deformation values for the start and end of the linear region are decreased in some of the groups. The value for the deformation at maximum load is decreased only in the 2-3 day group while the other groups are in the same range as the controls. The load values decrease significantly for the first three days after parturition followed by an increase in the 7-9 day group with values significantly higher than those for the non-pregnant animals. In the last post partum groups the load values are in the range of the control group. The "elastic stiffness" is significantly decreased in the 2-3 day group, rises sharply in the 7-9 day group and again decreases to the value of the non pregnant animals in the 12-13 day group.

Old multiparous rats The specimens invariably failed at one of the bone ligament junctions as in the young rats. The results from the biomechanical

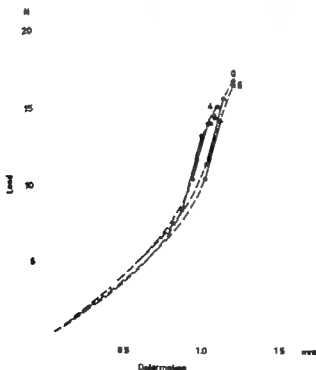


Fig. 50 Mean load-deformation curves of posterior cruciate ligament specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

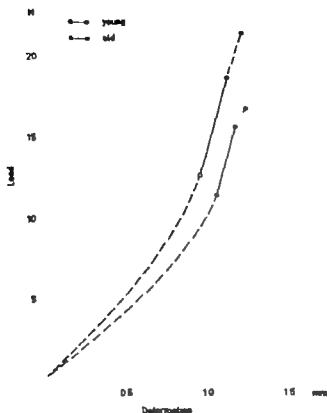


Fig 51 Mean load-deformation curves of posterior cruciate ligament specimens of young and old virgin rats.

tests (Fig 50 and Table A VIII) show no variation in the deformation values between the multiparous and virgin animals. Also the load and elastic stiffness values for the multiparous animals are not significantly different compared to the virgin rats.

Young and old virgin rats. The influence of aging on the biomechanical properties of the posterior cruciate ligament is shown in Fig. 51 and Table A VIII. The deformation values for the young animals are not different compared to those of the old group. The load values for the start and end of the linear region are not lower in the old virgins while the maximum load is significantly decreased in this group. The "elastic stiffness" tends to decrease in the old animals compared to the young ones. Thus the consequence of

Table XI Amount of collagen per millimeter specimen length of muscle tendons (mean values \pm S.E.M.) from young and old rats.

	No of animals	mg/mm
YOUNG		
Control	10	0.0217 ± 0.0018
Gestational		
Days		
6-10	2	0.0170 ± 0.0005
13-14	6	0.0190 ± 0.0026
15-16	7	0.0262 ± 0.0023
17-18	7	0.0243 ± 0.0021
19-20	7	0.0297 ± 0.0021
21	3	0.0279 ± 0.0044
Post partum		
Day		
4-6	3	0.0301 ± 0.0063
13-15	4	0.0375 ± 0.0051
17-20	8	$0.0361 \pm 0.0042^*$
25-38	3	0.0374 ± 0.0015
OLD		
N of pregnancies		
0=control	6	0.0281 ± 0.0060
4	14	0.0382 ± 0.0024
5	7	0.0360 ± 0.0024
6	3	0.0383 ± 0.0042
4+5+6	24	0.0381 ± 0.0015

* $2P < 0.05$ against control.

aging seems mainly to be reduced strength of the insertion of the ligament while the collagenous part is unaffected.

Muscle tendon

Young pregnant rats The specimens usually failed at the edge of one of the clamps because the pressure of the jaw edges weakens the tendon by thinning it out.

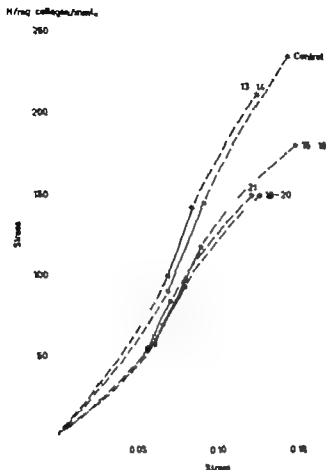


Fig 52. Mean stress-strain curves of muscle tendon specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

Compared to the non-pregnant group the amount of collagen per unit specimen length (Table XI) decreases in the 6-10 day gestational group followed by significantly increased values in the 19-20 day group. During the post partum phase all the values are significantly increased except for the 4-6 day group. Thus no sign of a return to non pregnant values during the involution period studied can be found in these muscle tendons.

The stress-strain relationships for the muscle tendons during pregnancy are presented in Figs 52 and 53 and Table A-IX. Both during gestation and in the

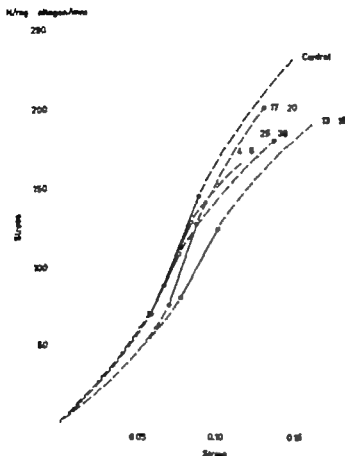


Fig. 53 Mean stress-strain curves of muscle tendon specimens of young rats during the post-partum period. The figures at the tops of the curves denote days after parturition.

post partum period the strain values for the start and end of the linear region are in the same range as for non-pregnant rats. The maximum deformation varies somewhat more probably due to the clamping of the specimens. The stress values of the linear region during gestation compared to the non-pregnant group are increased in the 6-10 day group. In the later gestational groups these values are not different from non-pregnant groups except for the 19-20 day and 21 day groups in which the values for the start of the linear region are significantly decreased. The maximum stress values show the same pat-

tern, although these values do not express the true ultimate tensile strength of the tendon. The "modulus of elasticity" in all gestational groups is not significantly different from the controls. During the post partum period the stress value for the start of the linear region in the 4-6 day group is significantly lower compared to the non pregnant animals. In the other post partum groups this value is in the same range as that of the controls. There are no differences in the values for the stress at the end of the linear region and the "modulus of elasticity" during involution compared to the non pregnant rats.

Old multiparous rats The specimens always failed at the edge of one of the

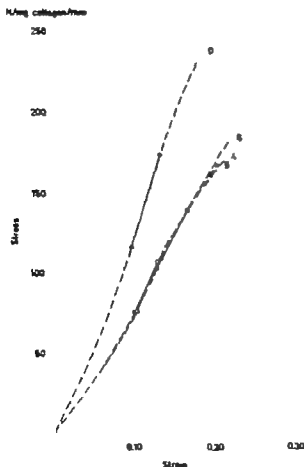


Fig 34 Mean stress-strain curves of muscle tendon specimens of old multiparous rats. The figures at the tops of the curves denote number of pregnancies.

clamps as did the tendons from the young animals. The amount of collagen per unit specimen length (Table XI) is not different between virginal and multiparous animals.

The values for the stress and strain at the start and end of the linear region and for the "modulus of elasticity" in the multiparous groups are not significantly different from the control group (Fig. 54 and Table A IX)

Young and old virginal rats There is no difference in the amount of collagen per unit specimen length between the two groups (Table XI). In the stress-strain relationship (Fig. 55 and Table A IX) the strain value for the

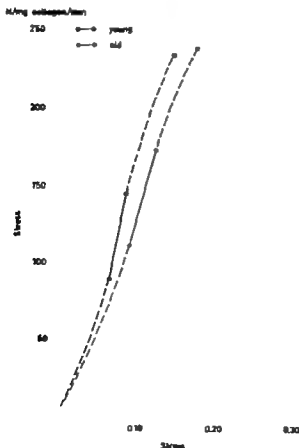


Fig. 55 Mean stress-strain curves of muscle tendon specimens of young and old virginal rats.

Table XII Amount of collagen per unit specimen length / skin specimens (mean values \pm S.E.M.) from young and old rats.

	No of animals	mg/mm
YOUNG		
Control	10	0.2557 \pm 0.0175
Gestational		
Days		
6-10	3	0.2266 \pm 0.0199
13-14	7	0.2744 \pm 0.0266
15-16	9	0.2916 \pm 0.0183
17-18	6	0.2373 \pm 0.0085
19-20	12	0.2541 \pm 0.0170
21	4	0.2659 \pm 0.0224
Post partum		
Days		
1-2	2	0.2745 \pm 0.0672
4-6	3	0.3000 \pm 0.0643
13-14	5	0.3473 \pm 0.0416
16-20	8	0.2866 \pm 0.0263
25-38	4	0.2431 \pm 0.0237
OLD		
No of pregnancies		
0=control	7	0.1299 \pm 0.0144
4	15	0.1475 \pm 0.0073
5	10	0.1815 \pm 0.0135
6	4	0.1343 \pm 0.0074
4+5+6	29	0.1570 \pm 0.0067

2P<0.10 2P<0.05 against young control.

2P<0.10, vs 2P<0.05 against old control

end of the linear region is significantly increased in the old group. The stress values show no differences nor do the "modulus of elasticity"

Skin

Young pregnant rats. The amount of collagen per unit specimen length (Table XII) does not change during gestation. In the post partum phase the

mean values for the different groups are in the range of the controls, although the value for the 13-14 day group tends to increase.

The results of the mechanical tests on the skin during pregnancy are given as diagrams and stress-strain parameters in Figs. 56 and 57 and Table A X. During gestation the strain values for the start and end of the linear region and the maximum strain are not significantly different from the non-pregnant group. The stress value for the start of the linear region tends to increase in the 6-10 day group and is significantly increased in the 13-14 day group. Then there is a decrease in this value to the same range as the non pregnant group. The stress value at the end of the linear region is significantly increased in the 6-10 day and 13-14 day groups, but from the 15-16 day group this value is at the non-pregnant level although the value tends to increase in the 21 day group. The "modulus of elasticity" is significantly increased in the 6-10 day group but only tends to increase in the 13-14 day group. The value then decreases to the range of the control group until the 19-20 day when it again

Mean collapse/sec

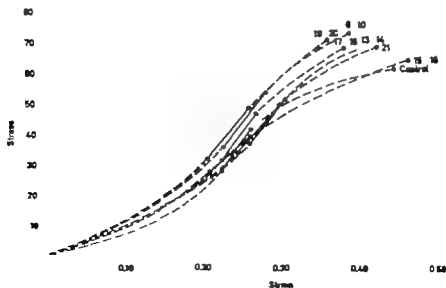


Fig. 56 Mean stress-strain curves of skin specimens of young rats during gestation. The figures at the tops of the curves denote days of gestation.

Table XII Amount of collagen per unit specimen length of skin specimens (mean values \pm S.E.M.) from young and old rats

	No of animals	mg/mm
YOUNG		
Control	10	0.2557 ± 0.0175
Gestational		
Days		
6-10	5	0.2266 ± 0.0199
13-14	7	0.2744 ± 0.0266
15-16	9	0.2916 ± 0.0183
17-18	6	0.2373 ± 0.0063
19-20	12	0.2541 ± 0.0170
21	4	0.2659 ± 0.0224
Post partum		
Days		
1-2	2	0.2743 ± 0.0672
4-6	3	0.3000 ± 0.0643
13-14	5	0.3473 ± 0.0416
16-20	8	0.2866 ± 0.0263
25-38	4	0.2431 ± 0.0237
OLD		
No of pregnancies		
0=control	7	$0.1289 \pm 0.0144^{**}$
4	13	0.1475 ± 0.0073
5	10	$0.1813 \pm 0.0135^{**}$
6	4	0.1343 ± 0.0074
4+5+6	29	0.1370 ± 0.0067

$2P < 0.10$ $2P < 0.05$ against young control.
 $\nabla 2P < 0.10$ $\nabla\nabla 2P < 0.05$ against old control.

end of the linear region is significantly increased in the old group. The stress values show no differences nor do the "modulus of elasticity"

Skin

Young pregnant rats The amount of collagen per unit specimen length (Table XII) does not change during gestation. In the post partum phase the

parturition. As reported in Part III the wet weight per unit length of rat tail tendons does not change during pregnancy and thus it might be assumed that there are different patterns of reactivity in the two types of tendons.

During gestation no changes are found in the strain values. The stress values are increased in the 6-10 day group and decreased but only at the start of the linear region in the last two gestational groups compared to the controls. During the post partum period all parameters are in the same range as the non-pregnant animals except for a decrease in the 4-6 day group. For the posterior cruciate ligament a decrease in load values is found during the early post partum period, while for the muscle tendon there is a decrease during late gestation. One explanation for this phenomenon might be an increase in ground substance or its water binding capacity in the tendon and the ligament. The collagen fibers are then pushed apart resulting in lower load and stress values and an increase in the visco-elastic properties. Bryant et al (1968) could not find any increase in the per cent of hexosamine in freeze-dried rat tail tendons studied almost daily in the period from six days before to eight days after parturition. However analysis of other ground substance components is not reported.

In multiparous animals the strain, stress and "modulus of elasticity" values are not different compared to the virginal animals. The amount of collagen per unit length is not affected by repeated pregnancies either. In old virginal animals the strain value at the linear region is increased compared to young ones, but all other biomechanical parameters as well as the amount of collagen per unit length are the same between the groups. The physical properties of the muscle tendon and also the collagenous part of the knee joint ligament are generally not changed due to increased age for the time period studied.

Skin

Young pregnant rats The amount of collagen per unit specimen length does not differ during pregnancy compared to the non-pregnant animals. This is somewhat contrary to Müller (1951) who found from histologic studies that during gestation the dermis in rat becomes thick and the collagen fibers are closely packed. Bryant et al. (1968) found an increased percentage of hydroxyproline in freeze-dried skin of rat studied in the period from 6 days before to 8 days after parturition.

The strain values are unchanged but the stress values are increased during the first half of gestation following which they return to the same range as the controls. These changes partly parallel those of the muscle tendons in which increased stress and unchanged strain values are found at the same period of gestation. In skin increased "modulus of elasticity" is also found close to parturition. Hormonal treatment of intact mature female rats has shown that both estradiol and progesterone increase the maximum load of skin as well as of tail tendons (Vogel, 1970). It is thus probable that these changes in the physical properties of collagenous tissues during pregnancy are mediated by sex hormones. It is also reasonable to assume that biomechanical parameters other than the above-mentioned can be influenced by these hormones. Pregnancy is a very complicated physiological process with intricate interactions, and deductive conclusions from model experiments where only one or a few factors have been analysed are thus uncertain.

Old multiparous rats The amount of collagen per unit specimen length is the same in multiparous as in virgin rats. During a single pregnancy there is no change in this parameter and this agrees well with the finding that there are no detectable cumulative changes.

The biomechanical properties of the collagenous framework are somewhat changed by repeated pregnancies manifested in increased strain values. The other parameters, however, show no differences between the multiparous and virgin rats. This results in decreased stiffness in the skin of the multiparous animals.

Young and old virgin rats Mendoza and Mulch (1964/65) Fry et al. (1964) and Vogel (1973) reported that the ultimate tensile strength increased with age. This is also confirmed in the present investigation. The strain values are unchanged but the stress and "modulus of elasticity" values are significantly increased in old virgin animals.

The collagenous framework of the skin becomes stronger during aging, and this process is only slightly modified by repeated pregnancies. The amount of collagen per unit specimen length decreases about 50 per cent in the old animals. During aging the collagen concentration in rat skin increases continuously as measured in per cent of wet weight (McGavack and Kao 1960). This is contrary to Vogel (1973) who found maximum concentration of hydroxyproline per gram fresh weight at the age of 4 months, followed by a slight decrease during aging. Fry et al. (1964) found a 40 per cent decrease

in the skin collagen concentration on wet weight basis, when comparing 10 and 20 months old rats. There is also slight reduction in the wet weight concentration of other proteins from about 8 months of age onward (McGavack and Kao 1960). The skin in old rats is thus somewhat atrophic but in spite of this its force resisting properties are more pronounced than in young rats.

THERMAL REACTIVITY IN RAT TAIL TENDONS

Introduction

It is an old observation that collagen melts and its fibrous structure shrinks to a rubbery one when exposed to heat. A collagen fiber heated to above 58 C shrinks to about one forth of its original length (Verzár 1964). The thermal shrinkage is a phase transition involving melting of crystalline regions (Flory and Garret, 1958). Physical means other than heat can also be used for studying the collagen fiber contraction. Banga et al. (1956) induced this phenomenon by immersing the tendon fiber in a 40 per cent solution of potassium iodide, while Elden and Webb (1961) used 8 molar urea. Other concentrated solutions of electrolytes have also been used. Standardized measurements of the physical events in this transformation of the collagenous structure can be performed in two ways (1) isotonically against a small counterweight that straightens the specimen but does not prevent shrinkage and (2) isometrically measuring the tension that develops. It was first shown by Verzár (1955) that the degree of thermal shrinkage was dependent on age tendon fibers from older rats tail tendons shrank more than those from younger ones and there was a fairly good correlation to increasing age. It has also been shown (Verzár 1964) that the tension developed in isometric thermal contraction increases with age. There are a great number of investigations in which the above mentioned methods have been used to study the "biological" age and aging of collagen fibers under varying experimental conditions, such as undernutrition (Chvapil and Hruza, 1959) ionizing radiation (Verzár 1964) and pregnancy (Árvey and Takács, 1964/65).

Young pregnant rats. Árvey and Takács (1964/65) measured the isotonic thermal contraction in rat tail tendons from animals divided in six age groups ranging between 4 and 13 months. Gestational animals from these groups were investigated and one of the groups was also studied on the 3-4 day after

parturition. On the 17 20 day of gestation increased tension was found in groups with animals aged 4 5 6 and 10 months. In the last group increased tension was found also on the 7 10 day of gestation. After parturition there was a reduction in the tension, but the mean value was still increased in comparison to the controls. They concluded that pregnancy enhanced the biological aging of collagen although this effect was reversible to a certain extent. Viidik et al. (1972) investigated tail tendons in five to six months old rats during the first 15 days of the post partum period with thermal isometric contraction test. The tension was increased compared to the non pregnant group and no tendency for a decrease during late post partum was found.

A few other studies to evaluate the distant effects of pregnancy on the physical properties of the collagenous framework have been made on the pubic symphysis (Storey 1957 Crelin and Brightman, 1957) dermis (Muller 1951) and bone (Currey and Hughes, 1973 Currey 1973) which was discussed in part II.

Old multiparous rats With increasing age the properties of collagen undergo changes resulting in increased resistance to physical procedures such as thermal denaturation, swelling in acid, solubility and digestion by enzymes. This has been attributed to an accumulation of covalent intermolecular cross-links (Verzár 1964 1969 Elden, 1964) In the rat tail tendon increased cross-linking with age has been demonstrated by Heikkinen and Kulonen (1964) and Steel and Everitt (1970)

Árvay et al. (1963) investigated thermal contraction of the tail tendon from 24 months old rats which had had about eight pregnancies. They found in the multiparous rats both the thermal contraction of tail tendons to be more pronounced and a lower amount of extractable collagen in comparison to virginal ones. The multiparous rats had an increased mortality at the age of 24 months, as 50 per cent of the original number of these animals were dead compared to 30 per cent in the virginal control group of the same age.

While it is well established that a pregnancy and the following involution period cause profound morphological changes in the prime target area, i.e. the genital tract (Cf Part I) the influence of this phenomenon on distant collagen is less evident (Cf Part II) Analysis of the time sequence of changes in a pregnancy cycle and the cumulative effects of such cycles, as reflected in the thermal reactivity of tail tendon fiber may give more information on

the behavior of distant collagen than biomechanical measurements because of its greater precision.

Materials and methods

Experimental procedure The tail was cut off at its base and in addition a small segment was removed from the distal end. Collagen fiber bundles were carefully pulled out in the distal direction, the skin remaining intact. The fibers were first weighed in sealed tubes to avoid evaporation and thereafter the lengths were measured after the fibers were straightened out but not stretched. The length of the fibers was about 7-8 cm and the weight 4-5 mg. The specimens were stored up to one hour in a tris buffered Ringer's solution, pH 7.4 at room temperature until the experiment.

The isometric contraction relaxation tests were performed in an apparatus described by Viidik (1968b). In this (Fig. 60) the tendon is mounted vertically between two clamps, the upper one suspended from a stainless steel blade via a rod on which the core of an inductive differential transformer is fastened (Bofors RRL-1 ± 3 mm). The lower clamp is fastened to an adjustable arm. With an appropriately stiff steel blade a displacement of the core by 0.2 mm or less per 10^{-3} N tension gives a satisfactory reading as a mV d.c. signal on a digital voltmeter (Olivon DPM 319) and ensures that the shortening is negligible in the 7.9 cm long fibers in these isometric experiments.

The specimen was mounted in the clamps and immersed into a buffered Ringer's solution, pH 7.4 at room temperature while an initial tension of 2×10^{-3} N was set. The clamping system was then immersed in the same type of Ringer's solution that was preheated to 62°C in a vessel placed in an outer waterbath and the temperature maintained constant. The tension was read versus time in tenths of minutes. Calibration was done before and after the experiments and constant results were obtained. The tension values were normated to fiber fresh weight per unit fiber length.

Results

Young pregnant rats The weight per unit length (mg/cm) of the tendon during the pregnancy is almost the same in all groups showing no significant differences (Table XIII).

Fig 60 Apparatus for measuring isometric thermal contraction in rat tail tendons. For explanation see text.



A typical contraction-relaxation curve for the mean of five tail tendons from a non pregnant rat is shown in Fig 61. The rapid contraction which

Table XIII Weight per unit length (mean value \pm S.E.M.) of tail tendons in young and old rats.

	No of animals	mg/cm
YOUNG		
Control	13	0.615 ± 0.028
Gestational		
Days		
6-10	3	0.595 ± 0.079
13-14	9	0.584 ± 0.023
15-16	9	0.563 ± 0.029
17-18	7	0.611 ± 0.037
19-20	13	0.579 ± 0.030
21	4	0.601 ± 0.047
Post partum		
Day		
1-2	3	0.575 ± 0.057
3-4	4	0.617 ± 0.037
5-7	3	0.602 ± 0.034
12-15	9	0.584 ± 0.042
16-20	9	0.645 ± 0.052
25-38	4	0.639 ± 0.088
OLD		
No of pregnancies		
0=control	9	0.739 ± 0.044
4	13	0.678 ± 0.036
5	10	0.628 ± 0.037^v
6	4	0.583 ± 0.033^{vv}
4+5+6	29	0.651 ± 0.024^{vv}

** $2P < 0.05$ against young control.

$2P < 0.10$ $^{vv} 2P < 0.05$ against old control.

ensues first is followed by a relaxation phase during a longer time interval. The tension at maximum contraction of the control animals was lower than that of the gestational rats Figs. 62 and 63 and Table A XI. During early gestation there is a significant increase in the isometric contraction tension followed by a decrease in the 13-14 day group to the same level as the controls. The values remain in this range until the 21st day when an increase is noted



Fig 61 Contraction-relaxation curve for the mean of five tail tendons of non-pregnant young rat. The tension values are normalized to milligrams fresh weight per cm tendon length and plotted against time.

again. The time to reach the maximum tension in the 6-10 day group is increased compared to the non pregnant group. After a decrease in the 13-14 day group to the range of the controls the value increases again in the 17-18 day and 19-20 day groups. In the 21 day group, however no increase is noted. One day after parturition the tension is not significantly different from the control group but increased tension values are found in the 3-4 day post partum group and in the rest of the post partum groups studied. Thus during the latter half of the involutional period no reversibility towards the non-pregnant values is seen. After parturition the time to reach the maximum tension is increased in the 3-4 day group but this is not seen in the 5-7 day group. In the last three post partum groups this parameter is increased and no return to the non-pregnant level is seen here either.

Old multiparous rats. The weight of the tendon per unit length for the pooled multiparous rats compared to the control group (Table XIII) tends

10 $\frac{m}{M}$

2000

1000

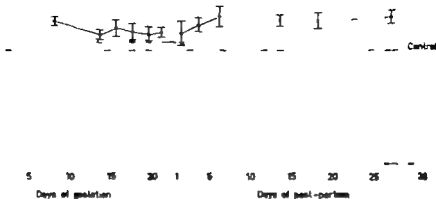


Fig. 62 Maximum isometric thermal contraction values for tail tendons of young rats during pregnancy. The tension values are normalized to milligram fresh weight per cm tendon length. (Mean values \pm S.E.M.)

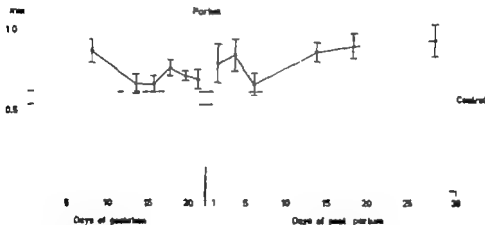


Fig. 63 Time to reach the maximum isometric thermal contraction values for tail tendons of young rats during pregnancy. (Mean values \pm S.E.M.)

to decrease and the value for the "6" pregnancies group is significantly lower.

The maximum tension and the time to reach this value is presented in Figs. 64 and 65 and Table A XI for the different groups. The maximum ten-

The time to reach maximum contraction is significantly increased for all

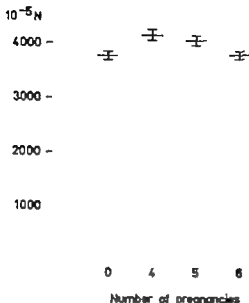


Fig. 64 Maximum isometric thermal contraction values for tail tendons of old multiparous rats. The tension values are normalized to milligram fresh weight per cm tendon length. (Mean values \pm 8 E.M.)

tion in the "4" pregnancies group is significantly increased, while the tension in the "5" pregnancies group only tends to increase. The value for the pooled multiparous rats is also significantly higher. The tension in the group with six previous pregnancies is almost the same as that of the controls. The greatest value is found in the "6" pregnancies group.

Young and old vaginal rats. In the old animals compared to the young ones the maximum contraction is almost three times greater (Figs. 61 and 63 and Table A XI) and the time to reach this value about four times longer (Figs. 62 and 64 and Table A XI).

Discussion

Young pregnant rats. The extragenital connective tissue does not fulfill any known physiological function connected with pregnancy. These collagenous

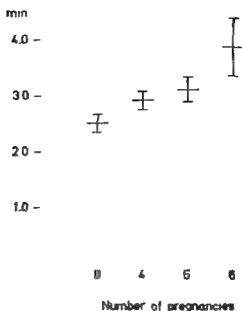


Fig. 65 Time to reach the maximum isometric contraction values of tail tendons of old multiparous rats. (Mean values \pm S.E.M.)

structures are not target tissues during gestation and the changes noted are less pronounced than in the genital tract. As discussed in connection with the influence of pregnancy on the biomechanical properties of skin, the changes that take place might be mediated via sex hormones, primarily estrogen and progesterone. Other hormones such as relaxin, corticosteroids and thyroxin might also be of importance. The relationship between estrogen and progesterone varies during pregnancy. The ovarian secretion of estrogen (Yoshimaga et al. 1969) and of progesterone (Hashimoto et al. 1968) have been studied in pregnant rats. The level of estrogen is relatively stable during gestation until a few days before parturition. It increases about 3-4 times and then rapidly decreases after parturition to almost a zero level. Progesterone is secreted in increasing amounts until the 14th day of gestation when a peak value is reached. During the late term progesterone secretion is decreased and reaches almost zero level just before parturition. The post partum period is dominated by progesterone secretion.

There might be a connection between the increased tension during early gestation and the post partum phase and the increased amount of progesterone. The slightly lower tension values from the 13th day of gestation to parturition might be explained by interaction of estrogen and progesterone. According to Vogel (1970) progesterone given to intact rats has a biphasic effect on the maximum tensile strength of rat tail tendons. Decreased values were noted during the first days of treatment followed by increased values. This was found both with high and low doses. Estrogen also resulted in increased tensile strength but without the decrease in the beginning (Vogel, 1970) Arvay and Takács (1965) administered estrogen and progesterone to intact rats for 16-17 days but could find no effect on tail tendons as tested by the thermal isotonic test. The knowledge of the action of sex hormones on extragenital connective tissue is insufficient to explain the changes found during pregnancy.

Old multiparous rats. Arvay et al. (1963) reported that tail tendons from old multiparous rats showed an enhanced isotonic contraction compared to virgins of the same age. However their animals came from a random pool of rats with an unknown number of previous pregnancies for each individual although the mean number of pregnancies for the pool was known. Only a few results from the older population were reported. In the present investigation the number of pregnancies for each rat is known and recorded.

The maximum tension for collagen fibers in isometric thermal reaction as well as the time to reach this point increase with age (e.g. Verrár 1963 1964 Viidik, 1969). This is also confirmed in the present investigation. The increased maximum contraction and time to reach this value is due to the enhanced crystalline structure of the collagen molecules, which requires a greater amount of energy and longer time to break the intra- and intermolecular bonds. Florey (1958) showed that an increased number of cross-links increases the denaturing temperature. Viidik (1969) demonstrated that a higher temperature breaks the cross-links more effectively than a lower temperature and a higher tension develops with identical collagen fiber specimens. The difference in maximum tension between the 4 pregnancies group and 5th and 6 pregnancies groups might be explained by the fact that a higher temperature than 62 °C is required for the complete denaturation of the specimen for the latter groups. The increased time required to reach maximum contraction supports this explanation.

The results show that pregnancy and the cumulative effect of repeated

tative but also qualitative changes had occurred in the collagenous framework of the cervix. In the vaginal wall specimens the size increased and amount of collagen per unit length decreased. The length but not deformation values were increased and the load values were reduced about three times during gestation. The strain values were not generally reduced. The stress values at the time of parturition were decreased to half the non-pregnant values, but the "modulus of elasticity" was not changed. Thus also in the vaginal wall changes in the force resisting properties of the collagenous framework were found.

In the genital tract during gestation quantitative and qualitative changes of the collagenous framework occurred that for the uterine horns could be called an adaptive process to the growing fetuses and for the uterine cervix and vagina an anticipation to the passage of the fetuses.

During the post partum period in the uterine horn specimens increased deformation and load values were recorded for the first two days after parturition followed by a decrease during the period, which biochemically is characterized by collagen catabolism. Later the values were restored towards the level of the non pregnant rats. The strain and stress values changed in the same way indicating derangements of the collagenous framework and loss of force resisting properties when the collagen was broken down. In the uterine cervix the deformation and load values also increased soon after parturition followed by a decrease but the load values at this time never reached the low level recorded just prior to parturition. The stress-strain relationship changed in the same way. A restoration towards the range of the non-pregnant animals was noted in the last post partum groups. The deformation values decreased somewhat but the load values of the vaginal wall specimens increased slightly after parturition and these values were not followed by the decrease noted for the other parts of the genital tract. The stress and strain values showed the same pattern as the load values reaching the non pregnant level in the last post partum groups. The collagenous framework of the vaginal wall was not subjected to as pronounced derangements as other parts of the genital tract during the involution period.

During gestation the physical properties of the pubic symphysis were changed. In the load-deformation curves a more pronounced toe-part appeared, the start of the linear region ensued later and the "elastic stiffness" was decreased compared to non-pregnant animals. During the first two weeks of the post partum period the properties of the pubic symphysis with some ex

ceptions remained unchanged compared to those of gestation. Then the values approached the range of the non-pregnant rats. The biomechanical properties of the posterior cruciate ligament were generally not affected during gestation but the load values were reduced for the first three days after parturition. They then increased significantly above the level of the non pregnant animals after the first week of the post partum period. In the last post-partum groups the values had returned to the range of those of the control group. Muscle tendons showed reduced stress values at the end of gestation. Also during the first week after parturition the stress values were reduced but during the rest of the involution period studied no changes were found compared to non-pregnant rats. In the skin specimens the stress values increased during the first half of gestation but then the values returned to the range of the non pregnant rats. Increased stress values were found in the first and last of the post partum groups studied. In the rest of the groups no changes were found.

Being associated with the process of parturition the biomechanical changes of the pubic symphysis might be considered as an anticipation to this process. After parturition the organ underwent a restoration to non-pregnant conditions. For the parallel fibered collagenous tissues changes were noted mainly around the time of parturition as decreased force resisting properties. In skin on the other hand a slight increase was noted at the same time. These changes might be a distant response to the profound changes of the collagenous framework of the genital tract but are without physiological significance.

Biomechanical investigations are limited in the sense that only fairly large changes occurring on the molecular level can be recorded. In the organs discussed above in addition to collagen there are other tissue components that might influence or modify the physical properties. The tail tendons of the rat consist of almost pure collagen and an estimation of the entropy of the fibers is possible by measuring its thermal reactivity. This method has a greater precision than tensile strength measurements and will allow safer conclusions of the behavior of the distant collagen during pregnancy. Analysis of the thermal isometric contraction of tail tendons from the same rats showed that the maximum contraction was increased or tended to increase in all except one group during gestation. The time to reach the maximum contraction value was increased during early and late gestation. During the post partum period the maximum contraction was increased in all groups except for the group

just after parturition. The time to reach the maximum contraction value was also not increased in the first group after parturition but increased in the rest of the groups during the post partum period. The entropy of the collagen in the tail tendons was generally increased during pregnancy. This supports the previously discussed findings that a distant collagen effect seems to occur.

As the restoration during the post partum period in some instances was incomplete the influence of repeated pregnancies on the physical properties of the connective tissue was also studied. The old multiparous rats had had 4-5 or 6 pregnancies, and were 22-23 months old when tested. They were matched against a virginal group of the same age.

The uterine horn specimens in the multiparous rats had decreased load values but the stress values were unchanged compared to the virgins. The strain values were reduced in the multiparous animals indicating a stiffer framework. A comparison between young and old virginal rats showed that in the old animals the load values were increased but the stress values were decreased. In the old multiparous rats but not in old virginal ones modulus of elasticity was decreased compared to young virgins. Thus aging qualitatively changes the collagenous framework and multiparity could to a certain degree influence this process.

The uterine cervix specimens of the multiparous animals had the same load and stress values as the old virgins. The strain values showed a tendency to increase in the multiparous rats. In old virginal rats compared to young ones the length values were increased, the load unchanged and the stress values decreased. In the old multiparous rats but not in the old virgins the "modulus of elasticity" was decreased compared to the young virginal rats, indicating the same pattern of aging as in the uterine horns.

Vaginal wall specimens of multiparous rats showed decreased maximum load and maximum stress values. These parameters were not different between young and old virginal rats. Repeated pregnancies but not aging influenced the force resisting properties of the collagenous framework here.

The influence of repeated pregnancies on the physical properties of extragenital collagenous structures was also investigated. In the pubic symphysis of multiparous rats the load values for the end of the linear region were decreased compared to virginal rats. No other biomechanical parameters were different. No differences were found between young and old virginal rats. In the posterior cruciate ligaments no differences were found between multipa-

rous and virginal rats. In old virginal rats the maximum load was decreased and the "elastic stiffness" tended to decrease compared to young virgins. As the ligament failed at one of its bone attachments the change in maximum load indicated changes in the bone-ligament junction. Age changes in the collagenous part of the ligament were small. Between young virginal and old multiparous rats differences were found with lower values for the start and end of the linear region and decreased "elastic stiffness" were noted in the old multiparous animals compared to the young virginal rats. Thus repeated pregnancies more than age seemed to change the physical properties for this ligament. In muscle tendons no changes were found between old multiparous and virginal rats. In old virginal rats compared to young ones the strain value for the end of the linear region was increased but the other parameters were not different. The strain values were increased and "modulus of elasticity" decreased for the multiparous rats compared to young virgins. Thus repeated pregnancies also influenced the physical properties of muscle tendons more than aging.

In skin specimens from old multiparous rats the strain values were increased compared to old virginal rats. The stress values were not different. In old virginal rats the stress values and "modulus of elasticity" were increased compared to young virgins. These parameters were also increased for old multiparous rats compared to the young virginal ones. Age changes in the skin of the rat were more pronounced than in other extragenital structures and it was thus difficult to assess the influence of repeated pregnancies.

In the old multiparous rats the maximum thermal contraction of tail tendons was increased compared to the virgins. This was also the case for the time to reach this value. In old virginal rats compared to young ones the maximum contraction and the time was increased very noticeably. Thus repeated pregnancies enhance the "biological aging" of the collagenous framework in the tail tendons.

It can be concluded that changes in the physical properties of the collagenous framework in the reproductive tract are part of an adaptive physiological process and the changes found can be interpreted in terms of this. On the other hand changes in the biomechanical properties of the extragenital connective tissue except for the pubic symphysis are more difficult to explain from a functional point of view. They seem to be a side effect distant to the functionally meaningful changes in the reproductive tract.

Repeated pregnancies resulted in changed biomechanical properties of both genital and extragenital collagenous structures. In several of the structures tested the results indicated enhanced "biological aging"

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APPENDIX

TABLE

[illegible]

TABLE A VII

Tensile strength parameters of the starling ovaris specimens (mean values \pm S.E.) from young and old sets
 TP 0.10 sec HF 0.03 against young control

Parameters	No. of animals	t_{L_0}	Δt_{L_0}	$\Delta t_{L_{max}}$	V_{L_0}	V_{L_0}	V_{L_0}	V_{max}	\tan
Demolition		mm	mm	mm	N	N	N	N	N/mm
YOUNG									
Control	13		3.5	5.1	8	0.4	5.7	5	5.5 0.7
Gastric/ovarian									
Days									
6 10	2	2.6 0.3	5.7 0.5	8 0.4	6.7 0.8	0.1 1.3	10.6	8	17 1.0
13 1		5.5 0.0	6.7 0.0	7.0 0	2.8 0	0.5	7 0.4	5.2	5.4
18 14		7.3 0.3	5 0.0	8.4 0.3	1.5 0.3	1.8 0.3	2 0.3	2.8 0.4	
7 18	5	6.5 0.3	6.7 0.3	13.1 0.3	5 1.0	4 0.1	0.6 0.3	0.3	0.1
20		12.3 0.0	1.8 0.3	13.8 0.3	0.1 0.0	0.3 0.0	0.6 0.1	0	1.0
21		13.0 1.3	13.6 1.3	13.8 1.1	0.1 0.0	0.3 0.0	0 0.1	0.2 0.0	
Post partum									
Days									
1	5	5 0.0	7.8 0.3	0.0	2 7	5.5 0.6	7.4 0.7	5.2 0.3	
2 2		0.0	7 0.0	8.2 0.3	2.5 0.0	3.2 0.1	4.3 0.3	3.8 0.3	
3		2 0.0	7	8.2 0.3	1 0.3	2 0.3	3.1 0.3	3.0 0.3	
8	3	4 0.0	7 0.0	8.3 1	1.6 0.3	2.1 0.3	2.6 0.3	2.3 0.3	
13 1	5	8.2 0.2	5 0.2	8.6 0	1.1 0.3	1.4 0.3	2.0 0.3	2.8 0.3	
5 16	2	2.9 1.1	1 1.1	2 1.1	1.7 0.0	2.3 0.3	2.7 0.1	5.1 0.3	
1 20		5 0.0	5 0.0	1 3	1.7 0.3	2.1 0.3	3.2 0.3	3.5 0.3	
21 20		5.6 0.0	8.0 0.0	8.8 0.0	1.9 0.0	2.6 0.0	3.0 0.3	5 1.3	
OLD									
No. of pregnancies									
control	5	8.1 0.0	5.2 0.3	8.2 0.3	8.1 1.1	5 1.3	8.8 2.3	8.3 1.8	
	18	8.8 0.2	8.8 0.3	8.8 0.4	7.1 1.0	6 1.3	13.8 2.3	9.3 1.3	
5	5	8.7 0.2	8.8 0.2	9 0.3	5.7 0.9	7.3 1.2	8.1 1.3	7.9 1.1	
8		8.8 0	8.8 0.7	8.3 0.7	8.8 1.8	8.6 1.6	11.1 2.0	8.6 2.8	
1-4	23	8.7 0.2	8.9 0.2	8.8 0.2	8.8 0	8 0.8	11 1.0	9.0 1.8	

TABLE A IV

ovule strength parameters of the uterine cervix specimens (mean values S.E.M.) from young and old rats
 XP 0.10 vs XP 00 against young control Y XP .10 Y XP 0.06 against old control

Parameter	σ_{Y_2}	σ_{Y_3}	$\sigma_{Y_{max}}$	I_0	I_0	I_0	$\sigma_{Y_{max}}$	$\sigma_{Y_{max}}$
Standardization								
YOUNG								
Control	1.5	0.36	0.03	0.34	0.04	0.03	0.06	0.03
Experimental								
Days								
0 18	2	0.27	0.04	0.30	0.07	0.23	0.09	0.03
1 1	5	23	0.09	.25	.09	0.23	0.09	0.03
16 18	7	0.20	0.04	0.23	0.09	.26	0.09	0.03
17 18		0.04	0.04	0.13	0.04	0.26	0.04	0.03
18 20		04	0.04	0.11	0.04	0.26	0.04	0.03
21	3	04	0.04	.13	0.04	.40	0.10	0.03
Post-partum								
Days								
2 3		0.26	0.03	0.24	0.03	0.43	0.06	0.03
5		0.27	0.04	0.27	0.04	.33	0.04	0.03
		0.27	0.04	0.43	0.04	0.27	0.14	.3
13 1		0.26	0.1	.46	0.13	0.43	0.04	0.2
15 16	2	0.23	0.04	.24	0.04	0.26	0.04	0.03
17 20		.15	0.04	0.23	0.04	.28	0.04	0.03
23 24		0.23	0.03	0.27	0.03	0.2	0.03	0.03
OLD		0.20	0.04	0.24	0.04	0.7	0.04	0.03
Post-partum								
Days								
0 control	6	0.25	0.03	0.26	0.03	0.43	0.06	0.03
18		0.21	0.03	0.26	0.03	0.7	0.04	0.03
6		0.24	0.04	.23	0.04	0.40	0.04	0.03
8		0.26	0.04	0.23	0.04	0.28	0.03	0.03
23		.24	0.03	0.23	0.03	0.43	0.03	0.03

TABLE A-VI I

Tensile strength parameters of the post-order associate ligaments (mean values \pm S.E.) from young and old rats
 III -18 vs 27% ES against young control

Parameter	$\Delta L_{1/2}$ mm		$\Delta L_{1/2}$ mm		$\Delta L_{1/2}$ mm		$F_{1/2}$ N		$F_{1/2}$ N		F_{max} N		E/m	
Control	mm		mm		mm		N		N		N		N/mm	
Young	12	0.95	0.87	2.11	0.06	3.13	0.96	13	0.7	13.8	1.0	21.7	0	1.0 1.8
Gonadotomized Rats	3	0.79	0.80 ⁰⁰	0.93	0.91 ⁰⁰	1.02	0.96 ⁰⁰	1	0.8	1.8	0.8	22.8	0.7	2.8 2.1
	8	0.90	0.09	2.11	0.00	1.18	0.18	13.0	0.8	18.8	0.8	18.8	0.8 ⁰⁰	30.0 1.8
	13	0.83	0.03	1.07	0.03	1.15	0.03	13.1	0	20.1	1.1	23.8	0.8	39 1.1
	18	0.93	0.04	1.05	0.04	1.15	0.04	13.8	2.3	18.8	1.1	21.6	1.3	39.8 1.4
	20	0.79	0.83	0.82	0.84 ⁰⁰	0.99	0.99	13.1	7	18.8	0.8	20.8	0.8	41.8 1
Post partum Rats	1	0.94	0.87	1.04	0.96	1.16	0.87	1.8	1.3	17.8	2.0	19.7	1.7	30.3 2.8
	2	0.88	0.13	0.98	0.13	1.1	0.09	9.2	1.0 ⁰⁰	13.1	1.1	1	0.8 ⁰⁰	33.2 4
Old	3	0.80	0.82 ⁰⁰	0.89	0.04 ⁰⁰	0.86	0.04 ⁰⁰	7.2	0.8	11	0.8 ⁰⁰	15.2	0	39.8 2 ⁰⁰
	8	0.78	0.88	0.81	0.87 ⁰⁰	1.07	0.8	11.7	1.1	18.2	1.6	22.7		3
	13	0.84	0.88 ⁰⁰	0.77	0.87 ⁰⁰	1.13	0.8	18.5	0.7 ⁰⁰	23.8	1.6 ⁰⁰	26.1	2.5 ⁰⁰	53.8 3
	18	1.00	0.80	1.11	0.08	1.84	0.42	13.2	1.8	16.1	0.6	24.0		5 7
	20	0.80	0.08	1.00	0.08	1.08	0.08	12	1.2	18	1.0	20	1.8	30.7 2.1
Old	23	1.13	0.80	1.20	0.08	1.34	0.08	18	2.3	16.1	2.2	33		1.1 3.1
	24													
No of programmes control	7	1.08	0.08	1.18	0.08	1.22	0.08	11	1.8	18.8	1.8	18.7	1.4 ⁰⁰	37.8 1.0 ⁰⁰
	14	0.84	0.03	1.08	0.08	1.12	0.08	18	0	1	15.1	8		25.8 1
	8	1.04	0.86	1.1	0.08	88	0.08	18	8	14.2	1	1	0.8	33.8 1.2
	8	0.80	0.80	1.82	0.80	1.87	0.08	1.1	1.3	1.3	1.0	2		34 2
	27	0.88	0.84	1.08	0.04	1.14	0.84	10.8	0.8	13	6	18.2	0	36.8 1.1

TABLE 1. IX

strength parameters of muscle endon specimens (mean values ± S.E.) from young and old rats									
age	sex	no. of animals	lg	lg	lg	lg	lg	lg	lg
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P	1P	2P
2P	2P	0.0° against young control	2P	1P	2P	0.5 against 1d control	2P		

TABLE

cell strength parameters of skin specimens (mean values) from young and old rats

Experiment	No. of animals	I_0	I_C	I_{Tmax}	I_0 n/mg/mm	I_C n/mg/mm	I_{Tmax} n/mg/mm	I_{Tmax} n/mg/mm
1	0 22	27	02	04	27 5	20 1	2 5	2 7
2	0 22	27	02	04	27 5	20 1	2 5	2 7
3	0 22	27	02	04	27 5	20 1	2 5	2 7
4	0 22	27	02	04	27 5	20 1	2 5	2 7
5	0 22	27	02	04	27 5	20 1	2 5	2 7
6	0 22	27	02	04	27 5	20 1	2 5	2 7
7	0 22	27	02	04	27 5	20 1	2 5	2 7
8	0 22	27	02	04	27 5	20 1	2 5	2 7
9	0 22	27	02	04	27 5	20 1	2 5	2 7
10	0 22	27	02	04	27 5	20 1	2 5	2 7
11	0 22	27	02	04	27 5	20 1	2 5	2 7
12	0 22	27	02	04	27 5	20 1	2 5	2 7
13	0 22	27	02	04	27 5	20 1	2 5	2 7
14	0 22	27	02	04	27 5	20 1	2 5	2 7
15	0 22	27	02	04	27 5	20 1	2 5	2 7
16	0 22	27	02	04	27 5	20 1	2 5	2 7
17	0 22	27	02	04	27 5	20 1	2 5	2 7
18	0 22	27	02	04	27 5	20 1	2 5	2 7
19	0 22	27	02	04	27 5	20 1	2 5	2 7
20	0 22	27	02	04	27 5	20 1	2 5	2 7
21	0 22	27	02	04	27 5	20 1	2 5	2 7
22	0 22	27	02	04	27 5	20 1	2 5	2 7
23	0 22	27	02	04	27 5	20 1	2 5	2 7
24	0 22	27	02	04	27 5	20 1	2 5	2 7
25	0 22	27	02	04	27 5	20 1	2 5	2 7
26	0 22	27	02	04	27 5	20 1	2 5	2 7
27	0 22	27	02	04	27 5	20 1	2 5	2 7
28	0 22	27	02	04	27 5	20 1	2 5	2 7
29	0 22	27	02	04	27 5	20 1	2 5	2 7
30	0 22	27	02	04	27 5	20 1	2 5	2 7
31	0 22	27	02	04	27 5	20 1	2 5	2 7
32	0 22	27	02	04	27 5	20 1	2 5	2 7
33	0 22	27	02	04	27 5	20 1	2 5	2 7
34	0 22	27	02	04	27 5	20 1	2 5	2 7
35	0 22	27	02	04	27 5	20 1	2 5	2 7
36	0 22	27	02	04	27 5	20 1	2 5	2 7
37	0 22	27	02	04	27 5	20 1	2 5	2 7
38	0 22	27	02	04	27 5	20 1	2 5	2 7
39	0 22	27	02	04	27 5	20 1	2 5	2 7
40	0 22	27	02	04	27 5	20 1	2 5	2 7
41	0 22	27	02	04	27 5	20 1	2 5	2 7
42	0 22	27	02	04	27 5	20 1	2 5	2 7
43	0 22	27	02	04	27 5	20 1	2 5	2 7
44	0 22	27	02	04	27 5	20 1	2 5	2 7
45	0 22	27	02	04	27 5	20 1	2 5	2 7
46	0 22	27	02	04	27 5	20 1	2 5	2 7
47	0 22	27	02	04	27 5	20 1	2 5	2 7
48	0 22	27	02	04	27 5	20 1	2 5	2 7
49	0 22	27	02	04	27 5	20 1	2 5	2 7
50	0 22	27	02	04	27 5	20 1	2 5	2 7
51	0 22	27	02	04	27 5	20 1	2 5	2 7
52	0 22	27	02	04	27 5	20 1	2 5	2 7
53	0 22	27	02	04	27 5	20 1	2 5	2 7
54	0 22	27	02	04	27 5	20 1	2 5	2 7
55	0 22	27	02	04	27 5	20 1	2 5	2 7
56	0 22	27	02	04	27 5	20 1	2 5	2 7
57	0 22	27	02	04	27 5	20 1	2 5	2 7
58	0 22	27	02	04	27 5	20 1	2 5	2 7
59	0 22	27	02	04	27 5	20 1	2 5	2 7
60	0 22	27	02	04	27 5	20 1	2 5	2 7
61	0 22	27	02	04	27 5	20 1	2 5	2 7
62	0 22	27	02	04	27 5	20 1	2 5	2 7
63	0 22	27	02	04	27 5	20 1	2 5	2 7
64	0 22	27	02	04	27 5	20 1	2 5	2 7
65	0 22	27	02	04	27 5	20 1	2 5	2 7
66	0 22	27	02	04	27 5	20 1	2 5	2 7
67	0 22	27	02	04	27 5	20 1	2 5	2 7
68	0 22	27	02	04	27 5	20 1	2 5	2 7
69	0 22	27	02	04	27 5	20 1	2 5	2 7
70	0 22	27	02	04	27 5	20 1	2 5	2 7
71	0 22	27	02	04	27 5	20 1	2 5	2 7
72	0 22	27	02	04	27 5	20 1	2 5	2 7
73	0 22	27	02	04	27 5	20 1	2 5	2 7
74	0 22	27	02	04	27 5	20 1	2 5	2 7
75	0 22	27	02	04	27 5	20 1	2 5	2 7
76	0 22	27	02	04	27 5	20 1	2 5	2 7
77	0 22	27	02	04	27 5	20 1	2 5	2 7
78	0 22	27	02	04	27 5	20 1	2 5	2 7
79	0 22	27	02	04	27 5	20 1	2 5	2 7
80	0 22	27	02	04	27 5	20 1	2 5	2 7
81	0 22	27	02	04	27 5	20 1	2 5	2 7
82	0 22	27	02	04	27 5	20 1	2 5	2 7
83	0 22	27	02	04	27 5	20 1	2 5	2 7
84	0 22	27	02	04	27 5	20 1	2 5	2 7
85	0 22	27	02	04	27 5	20 1	2 5	2 7
86	0 22	27	02	04	27 5	20 1	2 5	2 7
87	0 22	27	02	04	27 5	20 1	2 5	2 7
88	0 22	27	02	04	27 5	20 1	2 5	2 7
89	0 22	27	02	04	27 5	20 1	2 5	2 7
90	0 22	27	02	04	27 5	20 1	2 5	2 7
91	0 22	27	02	04	27 5	20 1	2 5	2 7
92	0 22	27	02	04	27 5	20 1	2 5	2 7
93	0 22	27	02	04	27 5	20 1	2 5	2 7
94	0 22	27	02	04	27 5	20 1	2 5	2 7
95	0 22	27	02	04	27 5	20 1	2 5	2 7
96	0 22	27	02	04	27 5	20 1	2 5	2 7
97	0 22	27	02	04	27 5	20 1	2 5	2 7
98	0 22	27	02	04	27 5	20 1	2 5	2 7
99	0 22	27	02	04	27 5	20 1	2 5	2 7
100	0 22	27	02	04	27 5	20 1	2 5	2 7

TABLE A XI

Thermal isometric contraction versus time (mean value \pm S E M)
of tail tendon in young and old rats

2P 0 10 2P 0 05 ag inst young control
 ♀ 2P 0 10 ♀♀ 2P 0 05 ag inst old control

(Tension is normalized to mg tendon fresh weight per
cm tendon length)

		No of animals	Maximum 10 ⁶ N	Tension Time mi
YOUNG				
Control		13	1430 \pm 50	0 58 \pm 0 04
Gestational Days				
6	10	3	1750 \pm 50	0 87 \pm 0 07
13	14	9	1579 \pm 63	0 67 \pm 0 06
15	16	9	1658 \pm 102	0 67 \pm 0 05
17	18	7	1620 \pm 96	0 77 \pm 0 05
19	20	13	1574 \pm 74	0 72 \pm 0 03
	21	4	1610 \pm 60	0 70 \pm 0 06
Post partum Days				
1	2	3	1593 \pm 137	0 80 \pm 0 12
3	4	4	1695 \pm 86	0 8 \pm 0 10
5	7	3	1800 \pm 120	0 67 \pm 0 07
12	15	9	1760 \pm 50	0 87 \pm 0 06
16	20	9	1760 \pm 80	0 91 \pm 0 08
25	28	4	1910 \pm 70	0 9 \pm 0 10
LD				
If pregnant				
0	control	3	376 \pm 77	2 52 \pm 0 16
		15	4147 \pm 92 ^{♀♀}	2 94 \pm 0 16 ^{♀♀}
		10	4018 \pm 107 [♀]	3 13 \pm 0 22 ^{♀♀}
			3759 \pm 76	3 90 \pm 0 52 ^{♀♀}
4 5		3	3972 \pm 60 ^{♀♀}	3 23 \pm 0 14 ^{♀♀}

ACTA PHYSIOLOGICA SCANDINAVICA

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CRITICAL STUDY OF HANSSON'S COBALT PHOSPHATE METHOD

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CARBONIC ANHYDRASE HISTOCHEMISTRY

A CRITICAL STUDY OF HANSSON'S COBALT-PHOSPHATE METHOD

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INTRODUCTION

The first published histochemical technique for demonstration of carbonic anhydrase was that of Kurata (1953). It has however been found to give unsatisfactory results by most workers (Hausler 1958, Fand et al 1959, Pearse 1968).

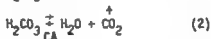
Hausler (1958) described a histochemical method with sections floating on the medium which was slightly modified by Waldayer and Hausler (1959). The validity of this method has been accepted by some (Korhonen and Korhonen 1965, Leder 1971) and denied by others (Mustakallio et al 1960, Arnold 1966, Muther 1972, Chung 1973). The long incubation times, 20-120 minutes, are a serious drawback since diffusion of the enzyme or the reaction product may take place. Inhibition of the staining requires millimolar concentrations of acetazolamide, an inhibitor with a K_i of about 10^{-8} M. This makes it doubtful if the inhibitor indeed acts in a specific manner and precludes its use as a check of the specificity of staining.

Hansson (1967) modified Hausler's method by adding phosphate to the medium. This changes the composition of the reaction product and

allows much shorter incubation times 1-15 min. A further and most important difference is that specific inhibitors of carbonic anhydrase at micromolar concentrations abolish the staining.

In Hansson's method sections are floated on the surface of a freshly prepared incubation medium containing NaHCO_3 , CoSO_4 , H_2O_4 and KH_2PO_4 . Increasing amounts of cobalt and phosphate accumulate in active sections during the incubation (Hansson 1967). However, the exact nature of the cobalt phosphate complex has not been clarified. By treatment with $(\text{NH}_4)_2\text{S}$ it is visualized as a black precipitate probably containing CoS . The deposition of cobalt and phosphate as well as the visible staining is inhibited by low concentrations of acetazolamide (Hansson 1967).

As discussed by Hansson, the dehydration of HCO_3^- to CO_2 and OH^- is the basis of the histochemical staining. Continuous local OH^- formation at sites of carbonic anhydrase activity causes deposition of the basic cobalt phosphate complex. The processes responsible for the alkalization can be written



The loss of CO_2 is the essential reaction which forces reactions 1 and 2 to the right, resulting in a production of OH^- . No staining occurs if the loss of CO_2 is prevented by keeping an atmosphere of CO_2 above the medium with the floating sections.

The phenomena close to the fluid surface are the important ones since the catalyzed staining reaction can occur only where there is CO_2 loss to the air. The sections remain unstained if they dip under the surface during the incubation (Hansson 1967).

Hansson's method has been used in a number of studies (Hansson 1968, Laurent et al 1969, Pesetsky 1969, Lauwers et al 1970, Rosen 1970, 1972 a, b, Muster and Rosen 1973 a, b, Rosen and Friedley 1973, Bhattacharjee 1971, 1972, Lönnnerholm 1971, 1972, 1973, 1974, Lönnnerholm and Ridderstråle 1974). Distinct and consistent staining patterns

were reported by these authors. Further available biochemical data were found to correlate well with the histochemical staining.

The method has been criticized by Muther (1972) however. He questions its specificity on the basis of a number of experiments some of which will be discussed in the course of the present paper. Others of his findings are dealt with in a counter critique by Rosen and Musser (1972). Lightfoot and Cassidy (1973) have also argued in favour of the validity of the method.

In our laboratory data have accumulated which strongly support the idea that Hansson's method is indeed specific for carbonic anhydrase activity. These findings are reported here.

GENERAL METHODS

TISSUE PREPARATION

Fresh tissues were used for biochemical assay. Tissues to be studied histochemically were frozen by immersion in isopentane cooled with liquid nitrogen with or without previous fixation. They were stored in small plastic bags at -70°C for days or weeks before use.

The fixative was 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The glutaraldehyde was prepared from 50% stock solution by on-stage vacuum distillation and the purity of the distillate was checked by ultraviolet spectrophotometry (Anderson 1967). Tissues (blocks, maximum thickness 4 mm) were immersed in the fixative for 16-20 hours at 4°C . They were then rinsed in 0.2 M cacodylate 0.05 M phosphate buffer (pH 7.4) to remove excess fixative. Some tissues were perfused with fixative *in vivo* (see below) with or without subsequent fixation by immersion.

Human kidney tissue was obtained from fresh donor kidneys prepared for transplantation by perfusion with cold Ring-dextran type F solution. They were stored at 4°C . The tissue preparation started within 24 hours. Further details on these kidneys are given by Lönnnerdal (1973).

For taste tissue was taken from 8 Wistar rats (300 g) brain and cervical spinal cord tissue from 4 male Sprague-Dawley rats (300-500 g) and kidneys from female Wistar rats (250-300 g). All animals were fed standard rat pellets and tap water *ad libitum*. They were anaesthetized with pentobarbital sodium (Mabumal[®] ACD) 40 mg/kg injected intraperitoneally. The tissues to be studied were perfused *in vivo* with saline for 5 min. The purpose was to remove the erythrocytes which contain large amounts of the enzyme. When fixed tissues were desired the perfusion with saline was followed by perfusion with the fixative for 10 min.

Spinny d gfi b Squalus anthias were captur d at the Swedish Atlantic co st in October and transported to th laborat ry in a tank with oxygen-bubbl d seawater They were used within 24 hrs of d live-ry One male and two females weighing 1.5 - 3 kg were studied They were ana thetized with MS 222^R (Sandoz) 100 mg/L of tank water A polyethylene tube was int oduced int rh dorsal aorta and the caudal part of the animal w s perfused with blood isotonic 3.3% NaCl fr 5-10 min Kidney ctal gland and skel tal muscle tis ue which all appeared blood fr s after the perfusion we then dissect d They were eithe homog nized for biochemical assay or frozen with or without pr vi s fixation for histochemical study

HISTOCHEMICAL STAINING PROCEDURE

Se ctions were cut in cryostat at 20°C

Se ctions f fixed tissue 8 µm or thick r were collected on cold 0.2 M sucrose 0.05 M phosphate buffer (pH 7.4) They were transferred to the incub ti n medium within 3 min

Se ctions f unfixed tissue could not be handled in thi way ince they disintegr t w th contact with th fluid The afore 15 µm thick se ctions were fr s-d i d for 1 hr at 0.03 mm Hg and 50°C and then incub ted These se ctions could b stored fr some days b fr use

When thinner se ction of eit fixed or unfixed tissues were desi ed they had to b supported by piece of Millipore^R filter (TH WP 75 µm thick pore size 0.45 µm Millipor Filter Corporation Bedford Mass USA) Freshly cut se ctions were thawed out piece of filter To ascertain good adhesion they were kept fr few min tes t room temper tur b fo the incubation

The staining procedur of Hansson (1967 1968) was used with only minor modifications Sections were flo ted n the surface of a medium containing CoSO₄ 1.75 mM H₂SO₄ 53 mM KH₂PO₄ 11.7 mM and NaHCO₃ 157 mM Thi concentr tion of KH₂PO₄ allows short incub ti n times of 1-15 min and we used thr oughout this study wh not therwis cited Th volume f the medium was 57 ml It was used immediately aft r the freshly prepared bicarbonate s lution (0.75 g NaHCO₃ in 40 ml d distilled v t) had been added to th oth r compon nts If drugs were added n the medium they were inc rp rated into the bicarbonat s lution b fore mixing

The pH f th medium i 5.8 immedi tely aft r mixing but increases due to f rmat ion and i of CO₂ int the al Th pH at th surf ce of the medium duri g the incubation i not known However an upp r limit is set by th pH f medium when it has (ft r several hours) eq lib rated with the i ; this v lue is 8.5 (Hansson 1967)

Se ctions not supported by Millipore^R filte s tend to disintegrate in th slightly aff res ant medium but were prote ted b re by addition of the non-ionic det rgent Tween 20^R t th medium in a final concentr ti n i 1/100 000 (v/v)

Afte the i cub ti n the se ctions were rinsed in saline buffered with phosphate (0.67 mM pH 5.9) They were then transferred to the freshly p epared bl eaking s lution 0.5% (NH₄)₂S in distilled water

Before use this solution was filtered through a filter paper where it had been deposited.

All solutions were kept at room temperature (22-24°C). The sections were transferred between the solutions by glass rod Petri dishes (diameter 9.5 cm) were used for all solutions. The choice of vessel for the incubation medium is of importance in the size of the surface area influencing the rate of CO_2 loss from the medium to the air.

The different steps of the staining procedure can be summarized

- 1) Float section on the incubation medium immediately after mixing. Make sure that the sections do not dip under the surface of the medium.
- 2) Float sections on the buffered line for 3 min.
- 3) Float sections on the blackening solution for 3 min.
- 4) Rinse for 1 min in three changes of line diluent.
- 5) Capture sections on slides and let them dry.
- 6) If desired count stain with a hematoxylin and eosin.
- 7) Dehydrate section through graded concentrations of ethanol, xylene and mount with Canada balsam.

When sections on Millipore filters are used the staining procedure has to be somewhat modified.

Ad 1 Allow the sections on their filter to equilibrate with the medium for 10 min under CO_2 atmosphere. This was accomplished by blowing 100% CO_2 over the medium with the following sections by means of an inverted funnel placed over the Petri dish. This prevents escape of CO_2 from the medium, which is the basis of the reaction. When the CO_2 atmosphere is removed the incubation starts. The sections must be on the top of the filter during the incubation.

Ad 2 3 4 Immerse the sections on their filter to secure adequate exposure of the sections to the solution.

Ad 5 Dehydrate and mount immediately after the final rinsing.

Ad 7 Dehydrate in 95% ethanol, absolute n-propanol, xylene and n-propanol (1:1) xylene and mount with Canada balsam. The filter becomes transparent during the dehydration which makes it possible to mount the sections together with their supporting filter.

INHIBITORS

Ibuprofen (Upjohn), tolazamide, methazolamide, Cl 13850 (American Cyanamid), ifenilamide (Chemapol), benznathiazide (Pfizer) and chlorthalidone (Mack Sharp and Bohme) were used without further purification. They were dissolved in water; benznathiazide it was necessary to add 2 moles of NaOH to each mole of inhibitor.

DETERMINATION OF ENZYME CONCENTRATIONS

The concentration of purified enzyme was measured spectrophotometrically using an extinction coefficient $\epsilon_{280}^{1\% 1\text{cm}}$ of 16.3 and 17.8.

f r HCA B and HCA C respectively (Gibbons and Edsall 1964) Apo-HCA B was assumed to have a similar value as HCA B. The molecular weight was taken to be 30 000 for all enzymes tested.

BIOCHEMICAL ASSAY OF CARBONIC ANHYDRASE ACTIVITY

Fresh tissues were weighed and homogenized by a Teflon^R plunger in glass tube with 9 parts of 0.25 M sucrose (dogfish tissues) or distilled water + 1 mM EDTA (rat prostate). The whole homogenates were used. The activity of the homogenates and of purified enzymes was determined by the changing pH method of Philpot and Philpot (1936) as described in detail by Maanen et al (1954). CO₂ is bubbled at constant rate through the reaction vessel containing phenol red. A standard amount of carbonate buffer is added which raises the pH and causes the indicator to change color. The hydration of CO₂ utilizes the added buffer base and causes the indicator to return to the original (acid) color within a certain time. One enzyme unit is defined as the activity which reduces this time by half. The activity of sample was calculated from 12 determinations on each of 3-4 dilutions of the sample. The assays were performed at 30-37°C.

I₅₀ DETERMINATIONS

The activities of the sulfonamides used (Table IV) were determined as their I₅₀-values at 30-37°C by the changing pH method according to Maanen et al (1954).

Pieces of human kidney cortex which had been stored at 70°C were thawed, weighed and homogenized with 9 parts of 0.25 M sucrose. After centrifugation at 100 000 x g and 4°C for 60 min the supernatant was removed and used for the assays. The amount of supernatant corresponding to 1 enzyme unit was determined. Twice that amount was then used and the concentration of drug yielded a reaction time corresponding to that of 1 enzyme unit was taken as the I₅₀-value. Each determination of I₅₀ in Table V is the average of 4-6 runs.

PHOTOMETRY

An objective but semi-quantitative comparison of the histochemical staining in the various sections was attempted by means of a photometer. Five sections of human kidney cortex which had been taken from the same piece of tissue and were of approximately the same size were used (for details see Table VI). This tissue contains structures with markedly different enzyme activities which gives an uneven distribution of the staining depth in the sections. To reference the photometer was set to measure the light which passed through the whole of the sections rather than through a small randomly located part. For this purpose the aperture of the photometer was set to barely exceed the width of the sections and was then left unchanged during the whole measuring procedure.

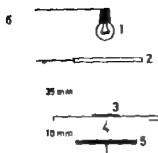


FIG 1 Construction of the photometer (not drawn to scale)

- 1) Stabilized light source
- 2) Opalacetic screen
- 3) Section on its lid
- 4) Adjustable part
- 5) Selenium barrier-layer cell with low resistance load. The current generated by the photocell is recorded by a galvanometer
- 6) Metal box (black). Its upper part can be removed

Test sections incubated in the presence of various sulfonamides were compared with unstained and fully stained (uninhibited) sections. The photometer generated a strong current with unstained than with fully stained sections since a light passed through the latter. The difference between the means of the unstained ($n=4$) and the fully stained ($n=5$) sections was calculated. Partial staining could then be calculated as a percentage of this difference. How differences in photometer readings relate to differences in staining intensity is not known in detail. However, the purpose of the present experiment was to compare numbers of sections in a semi-quantitative way. The apparatus used appeared to be adequate for this limited purpose.

RESULTS AND DISCUSSION

POSITION OF THE STAIN WITHIN THE THICKNESS OF THE SECTIONS

Rat kidney sections 8-20 μm thick were incubated in the standard medium. After the final rinse they were embedded in paraffin. Trans-sections of the original sections were then cut and mounted. The staining deposits were found to be located only at the upper surface of the sections, i.e. the surface which had been in contact with the air during the incubation (Fig. 2). As studied in detail by electron microscopy (Löfnerholm and Ridderstråle 1974) the stain penetrates with decreasing intensity only a few μm of tissue leaving the lower part of the sections unstained. Free floating sections and sections supported by Millipore^R filters were similarly stained. As described in Methods, free floating sections are floated on all solutions whereas sections on Millipore^R filters are floated on the incubation medium but immersed in the rinsing and blackening solutions. Thus, the uneven distribution of the staining is not an effect of the procedures following the incubation, i.e. rinsing and blackening.

The present findings on the distribution of the staining within the sections support the idea (see Introduction) that the phenomena at the surface of the incubation medium are the important ones. As one leaves the surface, CO_2 loss rapidly becomes rate limiting instead of dehydration of HCO_3^- .

The findings further indicate that pH-gradients caused by enzyme activity are established within the sections rather than in the medium. This should explain why local OH⁻ accumulations resulting in deposition of the basic cobalt phosphate complex can be maintained at enzyme sites in spite of the effervescence of the medium during the first minutes after mixing.

Hutner (1972) calculated that loss of CO_2 and not dehydration of HCO_3^- is the process which determines the rate of alkalization of the medium as a whole (my italics). He felt that this argued against



FIG. 2 Transverse section of 20 µm thick free floating section of fixed rat kidney stained for carbonic anhydrase activity. Incubation time 15 min. Note that the staining deposits are located only at the surface in contact with the air during the incubation. Arrows show erythrocytes inside vessels, where those situated at the surface are stained and those in the lower part of the section are unstained. $\times 1000$.

the mechanism of the staining outlined above. However, the present findings show that the analysis of the staining phenomena cannot rest on considerations concerning changes in the bulk of the medium. The same conclusion was drawn by Posen and Musser (1972) who found that a pH gradient rapidly develops in the medium (in the absence of sections) after mixing with a more alkaline pH close to the surface.

Since the histochemical staining reaction takes place in a micro milieu which is not easily accessible for a detailed chemical analysis, other kinds of experiments were designed for the present study of the specificity of the method.

PURIFIED ENZYME PREPARATIONS COMPARED BIOCHEMICALLY AND HISTOCHEMICALLY

Native enzymes. Low activity (HCA B) and high activity (HCA C) human erythrocyte carbonic anhydrase isoenzymes were prepared by ion exchange chromatography according to Nyman (1961). The activity of these purified enzymes was measured biochemically by the channing pH-method (see Methods) and in histochemical model experiments. In the latter, 5 µl droplets of serial dilutions (1:2) of the enzymes were deposited on small pieces of Millipore^R filter by an Eppendorf^R micropipette and

TABLE 1 The activities of purified HCA B and HCA C preparations as measured biochemically and in histochemical model experiments

Enzyme preparations	Biochemical method		Histochemical method	
	enzyme units/nmole of enzyme	relative activity $\frac{\text{HCA C}}{\text{HCA B}}$	smallest amount of enzyme producing detectable staining (pmoles)	relative activity $\frac{\text{HCA C}}{\text{HCA B}}$
1 HCA B	1.7		23	
HCA C	9.8	5.8	5.1	4.5
2. HCA B	1.3		26	
HCA C	9.3	7.2	4.0	6.5

allowed to dry in the air for 10 min. The pieces of filter were then incubated separately without any previous equilibration under CO_2 . The incubation time was always 9 min. After the final rinse in saline the stained spots on the filters (about 5 mm in diameter) were observed by the naked eye without mounting on slides. In this way the lowest concentration of the enzyme giving just visible staining was titrated. In the critical region 2-3 spots were read for each concentration.

Both isoenzymes produced staining in the histochemical model experiments; the minimal detectable amounts were about 25 pmoles for HCA B and 4.5 pmoles for HCA C (Table 1). Thus HCA C was roughly 5.5 times more active than HCA B. When samples of the same enzyme preparations were assayed biochemically HCA C was about 5.5 times as active as HCA B (Table 1).

In the histochemical test and in the changing pH method the enzyme operates under different conditions with respect to temperature (22-24 and 0°C), substrate (HCO_3^- and CO_2) and pH range (5.8-8.5 and 7.4-10.0).

At first glance it therefore seems surprising that the same relation between the activities of the enzymes is seen in the two tests. However this relation is rather constant also in kinetic measurements where the conditions have been varied to the same extent (Magid 1968 Wistrand et al 1974). It is only at pH values of 10 or more that the activity of HCA II approaches that of HCA C as a catalyst of the CO_2 hydration reaction (Khalifah 1971).

Modified enzymes Each molecule of carbonic anhydrase contains one zinc ion which can be reversibly removed from both HCA B and HCA C (Lindskog and Nyman 1964). The metal free apoenzyme has no catalytic activity. No important change of the protein structure seems to accompany the loss of the zinc ion (Lindskog and Malmström 1962 Wistrand and Rao 1968 Brewer et al 1968). A number of divalent metal ions bind to the active site of the apoenzyme. The addition of Zn^{2+} activates the enzyme completely and Co^{2+} restores about half of the activity but the Cu^{2+} -complex is inactive. After removal of these ions by dialysis catalytic activity can usually be restored by addition of Zn^{2+} indicating that denaturation of the enzyme has not occurred (Lindskog and Malmström 1962). In the present experiments metal free apoenzyme and Zn^{2+} , Co^{2+} and Cu^{2+} -enzymes were prepared from HCA B according to Lindskog and Nyman (1964). The biochemical assays showed that two experiments (1 & 2 in Table II) yielded apoenzymes with low residual activity. However the addition of Zn^{2+} restored less than half the activity indicating that inactivation of some enzyme had occurred. The third apoenzyme preparation showed considerable residual activity probably due to incomplete removal of the zinc ion. Reactivation with Zn^{2+} was complete.

The smallest amount of enzyme which produced detectable staining was titrated histochemically as described above. The relative activities were then calculated as percentage of that of the native enzyme to enable comparison with the biochemical findings. The histochemical and biochemical data were found to agree quite well (Table II) with a correlation coefficient r for the two sets of data of 0.91 ($p < 0.01$).

TABLE II The activities of various enzyme preparations as measured biochemically and in histochemical model experiments

Enzyme preparation	Activity as percentage of native enzyme	
	Biochemical method ^a	Histochemical method
1 Apoenzyme	1	< 5 ^b
Zn ²⁺ reactivated enzyme	22	16
2 Apoenzyme	1.4	3
Zn ²⁺ reactivated enzyme	43	52
3 Apoenzyme	22	27
Zn ²⁺ reactivated enzyme	110	84
Co ²⁺ enzyme	56	84
Cu ²⁺ enzyme	6	10

^aThe changing pH method was used as described in Method section except for experiment no. 3 where the assays were made in a stopped flow apparatus (see Wikstrand et al. 1974 for details) at 25°C and pH 7.05 using CO₂ as the substrate

^bMore concentrated enzyme not available

According to Lindsjö and Nyman (1964) Co²⁺ rapidly combines with metal free HCA B. Since the histochemical medium contains this ion some reactivation of the apoenzyme by formation of Co²⁺ enzyme during the incubation could be expected. However, the data of Table II show that no or little reactivation occurred, possibly because the composition of the histochemical medium is unfavourable.

In summary, the present findings show that the catalytic activity of various enzyme preparations as measured biochemically agrees well with the ability of the enzymes to produce staining in histochemical model experiments. This strongly supports the idea that the demonstration of carbonic anhydrase with Hansson's method depends on the catalytic activity of the enzyme.

UNCATALYZED AND CATALYZED STAINING

When inactive tissue sections are incubated long enough the spontaneous alkalization of the medium due to the uncatalyzed reaction $\text{HCO}_3^- \rightarrow \text{OH}^- + \text{CO}_2$ causes visible staining. Therefore the staining is specific and enzyme-dependent only if it occurs earlier than that of the uncatalyzed reaction. Hansson (1967) found that by changing the concentration of phosphate in the medium one could influence the time of appearance of both the catalyzed and uncatalyzed staining.

With the phosphate concentration used here 11.7 mM inactive sections remained unstained until after 20-25 min of incubation a diffuse staining appeared. This was seen in sections of a tissue lacking carbonic anhydrase i.e. dogfish skeletal muscle and in human kidney sections inactivated by heat see Table III. The staining was diffusely distributed except for the nuclei which generally appeared somewhat more stained than the other structures. This uncatalyzed staining was not influenced by addition of the inhibitors acetazolamide or ethoxzolamide (Table III).

Active uninhibited human kidney sections became stained already after 1-2 min of incubation. With increasing concentration of inhibitor in the medium the incubation times for visible staining were prolonged until they were similar to those of inactive sections. With 1 μM acetazolamide some tubular cells of the kidney sections became stained after 10-15 min of incubation (Table III) thus indicating considerable but not complete inhibition. Virtually total inhibition as evidenced by diffusely distributed staining appearing only after 20-25 min of incubation was seen with 10 μM acetazolamide and 1 μM ethoxzolamide (Table III). A few experiments with 10 times higher concentrations of these inhibitors gave similar results (data not shown).

The findings show that the incubation time should not exceed 15 min when the present high concentration of KH_2PO_4 in the medium is used. However with structures containing large amounts of the enzyme it might be necessary to use even shorter incubation times to prevent diffusion artifacts. These are easily recognized as areas of diffuse

TABLE III The histochemical staining of tissues where the enzyme activity is high (kidney) lacking (muscle) or inactivated by sulfonamides or heat

Tissue	Inhibitor in the medium	Incubation time (min)							
		3	6	10	15	20	25	30	
Dogfish muscle ^b									
	no inhibitor					(+)	+ ^a	+	
	acetazolamide 10 μM					(+)	+ ^a	+	
Heat inactivated human kidney cortex									
	no inhibitor					(+)	+ ^a	+	
	acetazolamide 10 μM					(+)	+ ^a	+	
	ethoxzolamide 1 μM					(+)	+ ^a	+	
Human kidney cortex									
	no inhibitor	+	+	+	+	+	+	+	
	acetazolamide 1 μM			(+)	+	+	+	+	
	10 μM					(+)	+ ^a	+	
	ethoxzolamide 1 μM					(+)	+ ^a	+	

positive = negative () weak and inconsistent staining Each point represents sections from at least 5 incubations 11.7 mM KH_2PO_4 in 11 incubation media 8 μ m thick of a floating sections of fixed tissues were used Unfixed kidney tissues were boiled for 10 min frozen and cut to give heat inactivated sections (8 μ m thick)

Some sections were unstained ^bHomogenates of dogfish muscle do not contain detectable enzyme activities as Table VII As shown in Fig. 11 the muscle fibers are inactive but one capillary shows how weak activity This capillary staining was neglected here

gray black staining surrounding the intensely stained sites. The most suitable time must be found for each structure.

Two conclusions can be drawn from the present findings:

- 1) The ability of the sulfonamides to inhibit the staining in active sections is due to their activity as inhibitors of carbonic anhydrase and not to unspecific interactions with the medium, since they do not influence the uncatalyzed staining in inactive sections.
- 2) The staining pattern in active tissues, for example the human kidney, is not caused by non-specific accumulation of the precipitate at some tissue components, since the staining caused by the uncatalyzed reaction in sections inactivated by the sulfonamides is diffusely distributed.

THE DEPOSITION OF PHOSPHATE (^{32}P) IN THE SECTIONS

The purpose of these experiments was to confirm the findings described in the previous paragraph by studying the catalyzed and uncatalyzed histochemical reactions by a quantitative method. Hansson (1967) found a parallel increase with time in the deposition of ^{60}Co and ^{32}P in active sections. There was a concomitant increase in the intensity of the staining. Muther (1972) also studied the ^{32}P uptake into sections and extended the incubation period to 60 min. He found a very slow initial uptake into kidney sections in the presence of acetazolamide $10\ \mu\text{M}$. However, the uptake rate increased after 30-40 min of incubation. He thought that this could be explained by an unspecific interaction of the inhibitor with the medium, possibly by complexing Co^{2+} . Since it was felt that Muther's interpretation is not correct, another purpose of the present experiments was to clarify the mechanism behind the uptake rates observed by him.

^{32}P labeled orthophosphate with approximately 10^{10} cpm/mole of phosphate was included in the medium at the usual concentration. Sections of human kidney cortex ($8\ \mu\text{m}$ thick, free floating) were incubated in the usual way. At timed intervals one section was removed from each batch of incubation medium. The sections were rinsed in the usual

TABLE III The histochemical staining of tissues where the enzyme activity is high (kidney) lacking (muscle) or inactivated by sulfonamides or heat

Tissue	Inhibitor in the medium	Incubation time (min)						
		3	5	10	15	20	25	30
Dogfish muscle ^b								
	no inhibitor					(+)	^a	+
	acetazolamide 10 μ M					(+)	^a	+
Heat inactivated human kidney cortex								
	no inhibitor					(+)	^a	+
	acetazolamide 10 μ M					(+)	^a	+
	ethoxzolamide 1 μ M					(+)	^a	+
Human kidney cortex								
	no inhibitor	+	+	+	+	+	+	+
	acetazolamide 1 μ M			(+)	+	+	^a	+
	10 μ M					(+)	^a	+
	ethoxzolamide 1 μ M					(+)	^a	+

+ positive - negative () = weak and inconsistent staining Each point represents sections from at least 5 incubations in 7 mM KH_2PO_4 in all incubation media 8 μ m thick of floating sections of fixed tissues used Unfixed kidney tissue was boiled for 10 min frozen and cut to give heat inactivated sections (8 μ m thick)

Some sections were unstained ^b Homogenates of dogfish muscle do not contain detectable enzyme activities see Table VII As shown in Fig. 11 the muscle fibers are inactive but some capillaries may show weak activity This capillary staining was neglected here

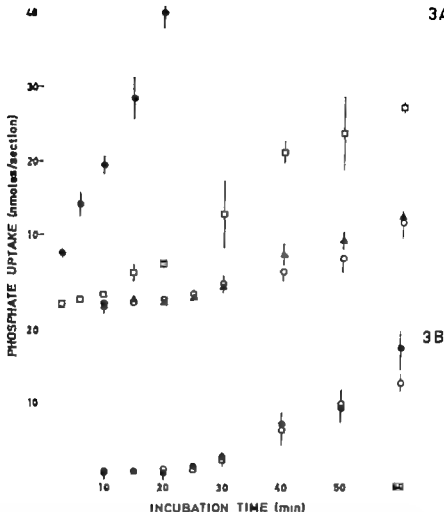


FIG 3 Deposition of phosphate in sections of human kidney cortex. Mean value ± 1 S.E. Statistical unit: sections. A) fixed tissue: (fixed) ● no inhibitor (n=4); □ taxolamide 1 μ M (n=3); ○ acetazolamide 10 μ M (n=6); ▲ thioxolamide 1 μ M (n=6). B) unfixed tissue (unfixed, boiled for 10 min): ● no inhibitor (n=5); ○ acetazolamide 10 μ M (n=5). All sections in A were cut from the same piece of tissue; all sections in B from another. The sections in A were somewhat smaller than those in B (about 0.5 and 0.8 cm^2 respectively).

for 16 hrs rinsed in sucrose for 2 hrs and frozen (see Method section for further details)

Tissue blocks were thawed weighed and homogenized with distilled water + 1 mM EDTA. The whole homogenates were assayed by the changing pH method. The activity of unfixed and fixed renal cortex was 195 ± 20.6 and 16.4 ± 3.4 enzyme units/g wet weight respectively (means of 3 samples ± 1 S.E.). Thus the activity of the fixed tissue was 8.4% of the unfixed similar to Muther's (1972) value for glutaraldehyde-fixed tissue.

20 μ m thick sections (n = 50) were cut and floated on 0.2 M sucrose + 0.05 M phosphate buffer (pH 7.4) at 22-24°C for 10 min. They were then homogenized and assayed as described above. Control sections cut from the same piece of tissue were homogenized immediately after cutting. In each experiment the activity of the floated sections was calculated as a percentage of the controls. The unfixed sections were found to retain only $7.2 \pm 1.6\%$ of their original enzyme activity whereas the fixed ones retained full activity $118 \pm 14.7\%$ (means of 3 experiments ± 1 S.E.).

These data would explain why fixed kidney sections show the same or a more intense staining than unfixed ones: the partial inactivation of the enzyme caused by the fixative is compensated for by the seemingly complete immobilization of the enzyme in the sections.

COMPARISON OF DIFFERENT SULFONAMIDES AS INHIBITORS OF CARBONIC ANHYDRASE AND AS INHIBITORS OF THE HISTOCHEMICAL STAINING

A comparison would seem to be difficult because the pH range covered is different in the histochemical and changing pH methods: 5.8-8.5 and 7.4-10.0 respectively. However both methods mainly utilize the pH range 6.5-9 where the activity of the sulfonamides is rather constant.

TABLE IV. CHEMICAL AND PHYSICAL PROPERTIES OF SULPHAMIDES WITH SUMMARY OF THEIR EFFECTS TOXICOLOGICAL AND METABOLICAL TESTS

NAME AND P.V.	STRUCTURE	$(pK_{a,2})$	ETHER PARTITION COEFF	TOXICOLOGICAL EFFECT	
				TOXICOLOGICAL TEST [SULPHAMYLUREA] ^a	TOXICOLOGICAL TEST (BASED FROM TOXICITY)
ETHIOZOLAMIDE 258		8.1	140	980	+++
ACETIZOLAMIDE 222		7.4 (9.1)	0.14	110	+++
PETIZOLAMIDE 224		7.2	0.62	180	+++
ISOKETIZOLAMIDE 152		7.4		280	+++
CHLOROKETIZOLAMIDE 226		6.7 (8.5)	0.04	57	++
SULFILLAMIDE 172		10.4	0.15	1	
CL 13659 274		7.5 (10.2)		NO EFFECT	NO EFFECT

^a FROM FURBER (1964), FURBER ET AL (1965). THE pK OF ISOKETIZOLAMIDE WAS SUPPLIED BY PYRIER.

^b THE RATIO OF CONCENTRATIONS IN ETHER AND PHOSPHATE BUFFERED SALINE (pH) FROM METERMAN ET AL (1964)

^c THE TOXICOLOGICAL DATA WERE CALCULATED FROM THE DATA OF TABLE . THE METABOLICAL ASSESSING SUPPLIED BY THE FINDING DATA IN P1 AND TABLE VI

(Taylor et al 1970 a) At a more acid pH the activity of all sulfonamides decreases equally probably due to titration of a group on the enzyme. Above pH 9 the activities of the individual sulfonamides are affected differently and this could possibly influence the results with the changing pH method. However Haren and Wiley (1968) found that I_{50} values of a large number of sulfonamides measured with this method in barbital buffer pH 7.4-7.9 are similar to those in carbonate buffer pH 7.4-10.0. Therefore the differences in pH range between the histochemical and changing pH methods as used here should not influence the activity of the sulfonamides sufficiently to preclude a comparison of their relative potency in the two methods.

Seven sulfonamides with widely different structures were selected for this study see Table IV.

The activities of different sulfonamides have been found to vary with the form of enzyme tested and particularly large differences have been observed between the high and low activity (with respect to CO_2) forms (Wistrand 1965 Taylor et al 1970 b).

It was therefore felt necessary to determine the inhibitory activities of the sulfonamides against the enzyme of the same tissue as tested histochemically. The activities were measured at $0 \pm 0.4^\circ C$ using the changing pH method. Inhibitor and enzyme were equilibrated for different times with and without the presence of CO_2 . Human renal cortex was used as enzyme source.

The results are seen in Table V. The sulfonamides were found to inhibit the human renal enzyme with I_{50} values similar to those previously found for the high activity forms of the human (Wistrand 1965) and dog (Haren and Wiley 1968) erythrocytes. This would agree with the evidence accumulated so far (see Wistrand et al 1974) that the major part of the activity of the human kidney depends on an enzyme which is similar to if not identical with the high activity human erythrocyte form HCA C. Five minutes of equilibration of enzyme and inhibitor in the presence of CO_2 increased the activities 2-4 fold and apparently so for all sulfonamides (cf 1st and 2nd columns of Table V). However incubating the thiazides with the enzyme in the absence of CO_2 increa

TABLE V The inhibition of carbonic anhydrase from human kidney cortex by different sulfonamides at 0.2 - 0.4°C

Drug	SEI		EI			
	Equilibration time (min)		Preincubation time (min)			
	0	5	1	5	30	60
Ethoxzolamide	0.011	0.003			0.004	
Acetazolamide	0.036	0.010			0.011	
Methazolamide	0.014	0.005			0.012	
Benzthiazide	2.6	1.3	0.086	0.033	0.006	0.006
Chlorothiazide	17	10	0.80	0.33	0.21	0.21
Sulfanilamide	2.4	1.4			1.2	
CI 13850	a	a			a	

The inhibition is expressed as 150% loss of the concentration of inhibitor (μ M) that reduced the enzyme activity by half (see Methods). SEI: enzyme and inhibitor were added in that order to the CO₂-bubbled reaction vessel. The timed reaction was started immediately (1st column) after 5 min of equilibration (2nd column).

EI: enzyme and inhibitor were preincubated until the reaction was 1 min at 0°C. They were then added to the CO₂-bubbled vessel and the reaction was started immediately.

a: No inhibitory effect at 50 μ M.

and their activities approximately 100 fold (cf. SEI and EI data for chlorothiazide and benzthiazide). No such effect was seen for the other sulfonamides. Why CO₂ (or HCO₃) prolongs the time of equilibration between the thiazides and the enzyme has not yet been settled (see Lindskog et al 1971). However, as shown by Haren and Wiley (1968) the thiazides can be made to equilibrate rapidly with the enzyme both in the presence (SEI) and absence (EI) of CO₂ if temperature is raised to 37°C.

In the histochemical test the intensity of the staining of fixed human kidney cortex sections was estimated either by the eye in the light microscope (Fig 6) or by photometry (Table VI)

Fig 6 summarizes the microscopic findings. Ethoxzolamide was found to be the most active drug inhibiting the histochemical staining completely even at the lowest concentration used 0.5 μ M. Acetazolamide and methazolamide were equally active and slightly more so than benzthiazide. Chlorothiazide was clearly less active than benzthiazide but more active than sulfanilamide. The control sulfonamide Cl 13850 had no activity at 50 μ M. Fig 7 shows an example of how the sections differed in staining intensities when incubated with different concentrations of chlorothiazide.

The estimation of the staining intensity of the sections by photometry agreed well with the subjective 6-min observations (Table VI).

The relative order of potency in the histochemical reaction corresponds well with that in the biochemical test particularly so if the inhibitors were allowed to equilibrate with the enzyme outside the reaction vessel (the EI data of Table V). This is understandable since in the histochemical reaction the temperature 22-24°C favours inhibition equilibrium. In some control experiments where the sections had been preincubated with the inhibitors without presence of bicarbonate (see legend to Fig 6) the results were similar to those of the standard experiments of Fig 6.

In a preliminary study (data not shown) unfixed human kidney sections (15 μ m thick freeze-dried) were tested. To obtain the same degree of inhibition somewhat higher concentrations of the sulfonamides were required than with fixed sections. The relative potencies of the sulfonamides were unchanged however.

In summary then as shown in Table IV the relative potencies of seven widely different sulfonamides as inhibitors of the histochemical staining were found to agree well with their relative potencies as inhibitors of carbonic anhydrase.

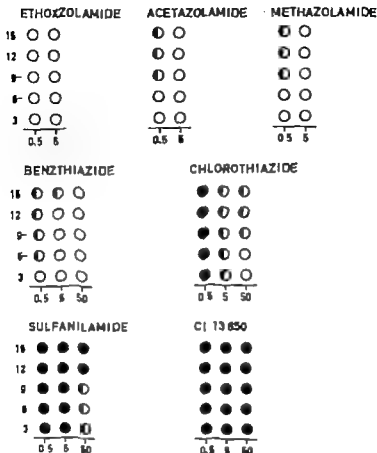


FIG 6 Effect of sulfonamide on the histochemical staining of sections of fixed human kidney cortex; light microcopy. Concentration of drug in the medium (μ M) on the x-axis. Incubation time (min) on the y-axis. ● no di. rubl. inhibition. ○ partial inhibition. ○ total inhibition. 8 μ m thick free floating sections which had been cut from the same piece of kidney tissue were incubated with each inhibitor at the different concentrations. Since these experiments were repeated three times each point shown represents 1 glc. section from 3 different incubations.

With acetazolamid, benzthiazid and chl. rothiazid sections were stained after preincubation with the inhibitor (data not shown). This was done by floating sections for 5 min on buffer (pH 7.4) sucrose solution (22-24°C) which contained the same concentration of inhibitor as the incubation medium.

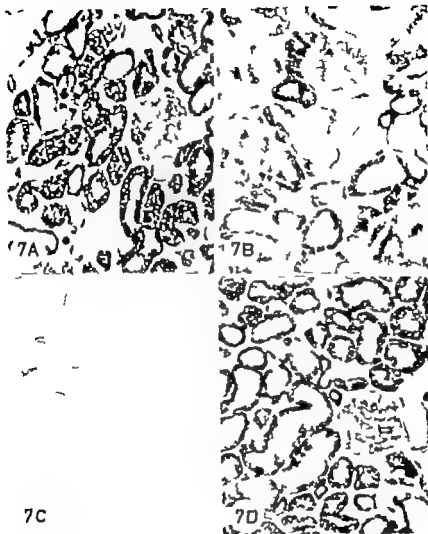


FIG 7 Effect of different concentration (A - 0.5 μ M B - 5 μ M C - 50 μ M) of chlorothiazide on the staining of human kidney sections D above control section incubated without inhibitor The sections are the same as used in Table VI $\times 180$

TABLE VI The effect of sulfonamides on the histochemical staining of sections of fixed human kidney cortex as measured photometrically

Drug	Concentration of drug in the medium (μ M)		
	0.5	5	50
Ethoxzolamide	1 (0.2)	3 (2.5)	
Acetazolamide	11 (0.17)	1 (0.2)	4 (0.7)
Methazolamide	4 (0.9)	2 (0.3)	4 (2.7)
Benzthiazide	57 (41-67)	11 (0.10)	1 (0.3)
Chlorothiazide	113 (102-121)	60 (57-62)	1 (0.3)
Sulfanilamide	111 (88-103)	111 (102-119)	69 (47-107)
CI 13850	117 (97-140)	96 (78-116)	132 (122-145)

The staining intensity of the sections is expressed as percentage of uninhibited controls (of tail see Methods). Mean values of 4 sections and standard error are shown. The incubation time was always 6 min. 8 μ m thick free floating sections were used and 11 sections were cut from the same piece of tissue.

ADMINISTRATION OF ACETAZOLAMIDE IN VIVO

Sodium acetazolamide (Diamox^R parenteral Lederle) dissolved in 0.9% NaCl was injected into the tail vein of male Sprague-Dawley rats weighing 300-400 g. In each experiment 4 rats were used. One rat was given saline only and the others 20, 40 and 100 mg/kg body weight of the drug respectively. The injected volume was always 1 ml/kg body weight. 30 min after the injection the kidneys were perfused (for details see L  nnerholm 1971) with saline for 3 min (4 experiments) or with fixative for 5 min (2 experiments) and then rapidly removed and frozen. Kidney sections were cut to include both cortex and medulla. Two sets of sections were prepared from each animal: one with free

floating sections (15 μ m thick) and another with sections on Millipore^R filters (4 or 15 μ m thick). Each set of sections was incubated in one dish and one section was removed after 2, 4, 6, 9 and 12 min respectively. Sections on Millipore^R filters were incubated without previous equilibration under CO₂. Free floating sections, fixed as well as unfixed, were freeze-dried and then incubated.

As seen in Fig. 8 A-D, the injected acetazolamide inhibited the staining in the kidney sections with a clear dose-response relationship. 20 mg/kg (Fig. 8 B) gave a somewhat delayed and weakened staining as compared to saline controls (Fig. 8 A). 40 mg/kg (Fig. 8 C) gave still weaker staining and 100 mg/kg (Fig. 8 D) inhibited all staining in some sections. Unfixed kidneys (photos not shown) showed a similar dose-response relationship.

The present findings agree with Hansson's (1967) report of delayed and weak staining in mouse kidney and gastric glands after 20 mg/kg acetazolamide intravenously. Bhattacharjee (1971) found inhibition of the staining of rabbit eye tissue after injection of 10 mg/kg of the same inhibitor.

We found it essential to handle the sections so as to avoid contact with any fluid before the timed incubation started. Thus, when the sections were floated on buffered sucrose for 10 min before transfer to the incubation medium, even 100 mg/kg of the drug failed to produce clearly visible inhibition (Fig. 8 E); the procedure had no effect on the staining of uninhibited control sections. These findings are not surprising since acetazolamide is a reversible inhibitor of the enzyme (Taylor et al. 1970 b). They might explain why Muther (1972)

FIG. 8 Effect of *in vivo* acetazolamide on the staining of rat kidneys. Fixed 4 μ m thick sections supported by Millipore^R filters. Incubation time 12 min. A: saline control; B: 20 mg/kg; C: 40 mg/kg; D: 100 mg/kg acetazolamide. E was cut from the same piece of kidney tissue as D, but the section was preincubated for 10 min in buffered sucrose before staining. All photographs are from the outer cortical zone. 180 (A) x 160 (B-E).



did not see any effect of injected acetazolamide 20 mg/kg on the staining of rat kidney sections the inhibitor was probably lost from the sections before the incubation since they were first collected in saline (unfixed sections) or floated on cold acetone (fixed sections)

CORRELATION BETWEEN HISTOCHEMICAL AND BIOCHEMICAL FINDINGS IN VARIOUS TISSUES

A number of tissues known to contain widely different amounts of carbonic anhydrase were chosen for a comparison between biochemical data and histochemical findings. They have been summarized in Table VII.

Manson and Fisher (1952) and Leiter (1964) reported high carbonic anhydrase activities in homogenates of the dorsolateral rat prostate but only low activities in the ventral prostate. This strikingly uneven distribution of the enzyme was confirmed here biochemically and histochemically. The dorsolateral rat prostate showed heavy staining of the whole glandular epithelium already after 1-3 min (Fig. 9 A). In the ventral prostate only scattered stained sites were seen even after incubation for 15 min (Fig. 9 B).

All vertebrates except sea going fishes seem to have abundant renal carbonic anhydrase (see Maren 1967 a). Its importance for urinary acidification is suggested by experiments with inhibitors of the enzyme (see Maren 1967 b, 1969). Histochemically high enzyme activity is found in the proximal and distal tubules as well as in the collecting duct in the mammalian kidney (Lönnnerholm 1971, 1973; Rosen 1972 a). Single species of aves, reptilia and amphibia have also been studied histochemically and clear enzyme activity was found in various parts of the kidney tubules (Rosen 1972 a; Lönnnerholm and Ridderström 1974).

In homogenates of the kidney of the spiny dogfish (Squalus acanthias) no or very low enzyme activity is found (Table VII). This correlates with an inability to vary the urinary pH and with a lack of effect on the urinary composition of acetazolamide in vivo (Hodler et



FIG 9 Dog prostate (A) and ventral (B) rat prostate. Fixed 8 μ m thick floating sections. Incubation time 3 min (A) 15 min (B). Lu = glandular lumen. Not intense uniform staining of the epithelium in A. In B only few stained sites in the otherwise inactive epithelium. The glandular structures are surrounded by an unstained interstitium. x 200

FIG 10 Dogfish kidney. Fixed 8 μ m thick floating sections. Incubation time 12 min (A) 9 min (B). Counterstaining with hematoxylin and eosin. (A) The kidney tubules are unstained except for single cells (rows) that these cells have basal position and do not reach the tubular lumen. In B stained glomeruli. In the upper left, the staining is not due to trapped erythrocytes. x 200

al 1955) Histochemically the tubular cells showed no enzyme activity except for some unidentified cells interspersed between them Fig 10A B These cells always had a basal position and were never found to reach the tubular lumen No attempt was made to study their distribution along the nephron The glomeruli were however stained Fig 10B The function of this enzyme in the glomeruli and the few stained cells of the kidney tubules is not clear at present However the finding that all tubular cells in contact with the urinary fluid lack carbonic anhydrase corresponds with the low activity of the homogenates and with the lack of effect of acetazolamide in vivo on the urinary composition It would also indicate that the ability to alter the urinary pH is associated with the presence of carbonic anhydrase in the kidney tubules since all vertebrates with ability to regulate the urinary pH have tubular enzyme (see above)

The dogfish rectal gland is a NaCl secreting organ and contains considerable amounts of carbonic anhydrase whereas no enzyme is found in the skeletal muscle Table VII This goes along with the present findings that this gland showed clear histochemical staining of the whole epithelium Fig 11 A whereas the muscle fibers were unstained Fig 11 B

Single nerve cells of the central nervous system of the rat were found by Giacobini (1961) to have a very low carbonic anhydrase activity 1/670th of that of erythrocytes The glia cells have 120 times

FIG 11 Dogfish rectal gland (A) and skeletal muscle (B) Fixed 8 μ m thick floating sections Incubation time 9 min (A) 12 min (B) The whole rectal gland epithelium is stained (A) The muscle fibers are unstained (B); arrow points to stained capillary x 300 (A) x 160 (B)

FIG 12 Rat cervical spinal cord (A) and choroid plexus (B) Fixed 4 μ m thick sections on Millipore filter Incubation time 15 min (A) 6 min (B) Note unstained triangularly shaped nerve cell in the center of A It is surrounded by stained structures possibly of glial origin B shows stained choroid plexus epithelium supported by unstained stroma 1000 (A) 400 (B)

FIG 13 Erythrocytes inside vessel in monkey kidney medulla Fixed 8 μ m thick free floating section Incubation time 2 min x 1000



13



TABLE VII Correlation between biochemical and histochemical tests for carbonic anhydrase activity in various tissues

Tissue	Species	Biochemistry		Histochemistry ^c
		enzyme units/g wet weight		
		present findings	literature data ^b	
dorsolateral prostate	rat	2670 (n=1)	2400	Fig. 8A
ventral		2 (n=1)	28	9B
kidney	dogfish	2 (n=3)	0-12	10A,B
rectal gland		210-216 (n=2)	204	11A
muscle		2 (n=1)	0	11B
nerve cells (CNS)	rat		4 ^d	12A
choroid plexus			900 ^d	12B
epithelium				
erythrocytes	various mammals	1400-2200	1100-2400	13
parietal cells	rat		7200	(11)
proximal tubules	man		490 ^g	(27)
distal			790 ^g	(27)
corneal endothelium	rabbit		250 (28)	(28)
epithelium			1 (28)	(28)
stroma				
lens			195	+ (3)
ciliary processes			23	§ (3,11)
retina			50	§ (2,40)
duodenum	rat		77	§ (11)
bladder mucosa	turtle		24 (54)	+§ (49)
skin	frog		74 ^f (52)	§ (52)

^aEach homogenate tested represents one 1mm³

^bReferences not found: Haren (1967b) are given with the data

^cFig. numbers refer to the present study. Figures in brackets are references to literature data: staining, no staining

^dCalculated from Giacobini (1961) assuming a value of 2400 e.u./g rat erythrocytes

^eCalculated from Lee and Mattenheimer (1964) assuming a value of 1400 e.u./g human erythrocytes

^fE.u./g dry weight

^gOnly some cells were stained

and the choroid plexus cells 250 times higher activity than the nerve cells respectively. This offered a possibility to compare biochemical and histochemical findings in cells rather than in whole tissues.

Rat brain and cervical spinal cord were studied histochemically. The staining pattern in unfixed sections could not be interpreted due to badly preserved tissue structure. In fixed sections however the large pyramidal cells in the cerebral cortex, the Purkinje's cells in the cerebellum and the large motor neurons in the ventral horn of the spinal cord could be identified with certainty. These nerve cells showed no enzyme activity (Fig. 12 A). They were surrounded with distinctly stained structures which could not be identified but may represent glia. In the choroid plexus the surface epithelium was intensely stained whereas the supporting stroma was unstained (Fig. 12 B).

The erythrocytes of all mammalian species tested contain large amounts of carbonic anhydrase (Table VII). Histochemically the erythrocytes seen in fixed tissues of man, monkey, rabbit, rat and mouse were heavily stained after short incubation times of 1-2 min (Fig. 13). In unfixed tissues however they generally failed to produce distinct staining. In an effort to clarify this discrepancy a droplet of erythrocytes freshly suspended in saline was deposited on a Millipore^R filter, dried and incubated. The staining was diffusely distributed as a precipitate on the filter paper as previously reported also by Bhattacharjee (1971). It is probably due to loss of enzyme from the disrupted unfixed cells during the incubation.

Previous studies of various tissues with Hansson's method have also demonstrated that the histochemical findings agree with data on enzyme activity obtained by other methods (see Table VII). Thus in the cow and rabbit cornea only the endothelial cells are histochemically active which correlates with biochemical data on dissected parts of these corneas (Lönnerholm 1972, 1974; Silverman and Garster 1973). The lens, ciliary body and retina show enzyme activity biochemically (see Maren 1967 b) and histochemically (Hansson 1968; Bhattacharjee 1971; Musser and Rosen 1973 a; Lönnerholm unpublished observations) in several mammalian species. Hansson (1968) demonstrated carbonic anhydrase activity

ty in several actively transporting cells known or suspected to have the enzyme among them parietal cells and duodenal and gall bladder epithelial cells. In the turtle urinary bladder mucosa and the frog skin the histochemical demonstration of single active cells corresponds with the findings of low enzyme activities in homogenates of these tissues (Rosen 1970 Scott et al 1970 Rosen and Friedly 1973)

In summary the present and previous studies show an excellent correlation between histochemical and biochemical findings. This is strong evidence in support of the validity of the histochemical method.

SUMMARY

The histochemical method of Hansson which is based on the precipitation of a cobalt phosphate complex was found to be specific for carbonic anhydrase by the following criteria:

- 1) The activity of purified enzyme preparations as measured biochemically was found to agree well with their ability to produce staining in histochemical model experiments. The human erythrocyte isoenzyme HCA C was clearly more active than the isoenzyme HCA B in both methods. The staining was also well related to the catalytic activity of enzymes modified by introduction of various metal ions at the active site (metal free apo-HCA B Cu^{2+} Co^{2+} and Zn^{2+} HCA B).
- 2) The carbonic anhydrase inhibitors acetazolamide and ethoxzolamide in micromolar concentrations inhibited the catalyzed staining in active sections. They did not influence the uncatalyzed staining which appears after long incubation in inactive sections (heat inactivated or from tissues lacking enzyme) nor did acetazolamide influence the precipitation of phosphate (^{32}P) in inactive sections. These findings indicate that the inhibitors do not interact critically with any component of the incubation medium but inhibit the histochemical staining in a specific manner.

- 3) The relative potency of structurally highly different sulfonamides as inhibitors of the histochemical staining reaction was found to correlate well with the inhibitory effect of these drugs as determined kinetically
- 4) Intravenous injection of physiologically active doses of acetazolamide 20-100 mg/kg to rats inhibited the histochemical staining with a clear dose response relationship
- 5) Histochemical demonstration of carbonic anhydrase in various tissues was found to correspond well with biochemical data. Both methods show high activity in erythrocytes of several species: rat dorsolateral prostate, rat choroid plexus epithelium and dogfish rectal gland. No or low activity is found in rat ventral prostate, nerve cells of rat CNS and dogfish muscle.
- 6) Histochemically the tubular cells of the dogfish kidney showed no enzyme activity except for a few unidentified cells. This corresponds with the low activities found in homogenates and with the lack of effect of acetazolamide in vivo on the urinary composition.

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THE VOLUNTARY CONTROL IN HUMAN BREATHING

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HELSINKI 1974

Hämeen Kirjapaino Oy
Tampere 1974

PREFACE

This investigation was carried out at the Institute of Physiology University of Helsinki, and at the Department of Pulmonary Diseases University Central Hospital, Helsinki.

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Helsinki, May 1974

Pekka Halttunen

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1. INTRODUCTION

Breathing is controlled both by autonomic and voluntary centers. A close relationship has been found earlier between the electrical and mechanical activity of the human respiratory muscles (VILJANEN 1967). Furthermore, in animal experiments, VILJANEN (1970, 1972) has shown a close linear relationship between the phrenic multifiber activity and the corresponding electromyogram impulse activity registered from the diaphragm. Likewise in preliminary investigations indications of a relationship between the subjective sensation and the electrical activity of the respiratory muscles (VILJANEN 1970, 1972) and the mechanical activity of the respiratory muscles (BERGSTROM, HALTTUNEN & VILJANEN 1972) have been found.

Since the available information does not adequately clarify the physiological mechanism underlying the voluntary control of breathing, it is appropriate to investigate this matter by a sensory-physiological measuring method, which is considered to highlight the gross activity of the higher centers of the brain. This calls for an investigation in which relationships between the subjective sensation and mechanical activity of the respiratory muscles and subjective sensation and electrical activity of the respiratory muscles are studied as well as the increased effect of the carbon dioxide stimulation on the said relationship. The latter partial investigation can be considered to clarify further the role autonomic control plays in the voluntary control.

In the present study the following problems are studied.

The relationship between the subjective sensation and the volumes produced by the respiratory muscles when they are controlled voluntarily

The relationship between the subjective sensation and the electrical activity produced by the intercostal muscles during voluntary inspiration

The effect of an increased carbon dioxide stimulation on the relationship between subjective sensation and respiratory volumes.

2 REVIEW OF LITERATURE

2.1. *Voluntary control of skeletal muscles*

In 1930 in experiments on the skeletal muscles REENPAA (RENVIST 1930) found a distinct relationship between the physical force of muscle (force force impulse) and the corresponding sensation quantity. Later a linear relationship was demonstrated between the force produced by the skeletal muscles and the corresponding electromyogram impulse frequency (JALAVISTO et al. 1939). A similar relationship has also been found between the number (Periodenzahl) of impulses registered from the voluntary muscle and the corresponding physical force impulse (BERGSTROM 1959). Furthermore BERGSTROM (1957, 1958) in experiments on the human skeletal muscles, has compared sensation quantity to electrical activity of the muscle on one hand, and to the mechanical activity on the other hand, and found that both electrical and mechanical parameters demonstrate well the subjective contents which control muscular function, this sensation being best represented by the number of EMG impulses. It is to be assumed that the subjective contents represent the macro state of the organic process of the brain (BERGSTROM 1973).

STEVENS (1959) has extensively investigated with psychophysical procedures the correlation between the magnitude of sensation and the corresponding stimulus quantity with various senses. He separates three classes of sensory scales in psychophysics.

1 Discriminability scales. These are constructed in the tradition of Fechner whose century-old dogma is that the sensation intensity grows as the logarithm of stimulus intensity grows (The Fechner law)

According to this

$$y = b \log x$$

where y is the magnitude of sensation, x the intensity of stimulus and b is a constant. This function produces a straight line in a semilog coordinate system y plotted against $\log x$.

Some measure of jnd, variability confusion, or resolving power is employed as a unit, and a scale is constructed by counting off such units.

2. Category scales (partition scales) These are constructed by one or another variation of the procedure that PLATEAU invented when he required observers to divide a segment of a continuum into equal appearing intervals. According to STEVENS (1959) the results in a category scale do not follow any simple function

3. Magnitude scale Here the observer simply estimates the apparent strength or intensity of his subjective impressions relative to a standard set either by himself or by the experimenter. He found that human subjects are able to make consistent estimations of the subjective quantities. In addition various senses followed the same function the power law when subjective magnitude is expressed as a function of the objective magnitude. This function forms a straight line in log log coordinate system

$$y = ax^b \rightarrow \log y = \log a + b \log x$$

Later this procedure has also been used for investigating the relationships between sensation quantity and the corresponding physical force in the skeletal muscles. A distinct relationship yielding the power function with an exponent value of 1.7 (STEVENS & MACK 1959) was found between the force of handgrip and the experience of force exerted. EISLER (1982) studied, using the same method, the relationship between the muscular force of the leg and the corresponding sensation quantity and obtained similar results

In the experiments on human respiratory muscles, also relationships between the sensation quantity in breathing and the number of EMG impulses from the respiratory muscles (VILJANEN 1970) as well as between sensation quantity and the EMG impulse frequency have been observed (VILJANEN 1972). A clear relationship has also been verified in preliminary experiments between subjective sensation and the volume produced by the respiratory muscles (BERGSTROM HALTTUNEN & VILJANEN 1972).

2.2. The neural control of respiratory muscles

In earlier studies on the neural control in human breathing several relationships have been verified between the electrical activity and the mechanical activity of the respiratory muscles during voluntary inspiration (VILJANEN 1967).

1. The total EMG impulse number bears a linear relationship during the course of inspiration to the first time integral of the inspiratory muscular work, the so-called physical action.

2. The EMG impulse frequency registered at the end of inspiration bears a linear relationship to the inspiratory muscular work.

3. Four seconds after the end of inspiration with the subject holding his breath the EMG impulse frequency was in direct proportion to the muscular force.

4. During one and the same inspiration the EMG impulse number is directly proportional to the pressure impulse. According to this the EMG impulse frequency during inspiration is directly proportional to the inspiratory muscle force.

BERGSTROM and KERTTULA (1961) have found in animal experiments that the linear relationship between the number of EMG impulses registered from the intercostal muscles and the corresponding mechanical activity remained the same during carbon dioxide stimulation. Likewise VILJANEN (1967) found in his experiments on man that the linear correlation between the number of EMG impulses and physical action of inspiration remains the same during CO₂ stimulation but the regression

coefficient changes so that the equivalent physical action can be produced by a smaller number of impulses.

The EMG activity registered from the diaphragm has been found to be directly proportional to the corresponding activity of the intercostal muscles during inspiration (VILJANEN & HALTTUNEN 1967 VILJANEN 1972) In animal experiments a nearly linear relation has been found between unit activity of the phrenic nerve and the electrical activity of the diaphragm (DITTLER & GARTEN 1912, GASSER & NEWCOMER 1921) The same linearity has also been demonstrated between the multi fiber activity of the phrenic nerve and the electrical activity of the diaphragm (VILJANEN 1970 1972) Furthermore, in animal experiments a close relationship has been observed between the afferent neural drive and the efferent electrical activity of the respiratory centers (VILJANEN 1970 1972)

Even though the diaphragm and the intercostal muscles are controlled by the same regulatory system they have been found to have partly at least, a different regulation. Their interactivity can be voluntarily controlled (VILJANEN & POP PIUS 1968 VILJANEN 1970 1972) Further in experiments on the cat, as the level of anaesthesia deepens, the electrical activity of the intercostal muscles decreases more than that of the diaphragm (VILJANEN et al 1968 VILJANEN 1970) The latter fact would support the notion that the diaphragm is regulated by a considerably more primitive and more powerful autonomic center than the intercostal muscles. On the other hand the number of muscle spindles in the intercostals has been established to be greater than that of the diaphragm (DOGIEL 1902, GREGOR 1904 HINSLEY et al. 1939 WINCLER & DELALOYE 1957) This may suggest that the intercostal muscles can give more afferent input to respiratory centers, and this feedback is more intensively controlled also by the higher respiratory centers than the diaphragm while the latter may be more primitive and intensively controlled by the autonomic centers. It has been established earlier that the muscle spindles in the intercostal muscles and their reflexes play an important part in the regulation of respiration (CAMPBELL & HOWELL 1902, VON EULER 1906 SEARS & NEWSOM DAVIS 1968)

3. THE RELATIONSHIP BETWEEN THE SUBJECTIVE MAGNITUDE AND THE VOLUME OF INSPIRED AND EXPIRED AIR PRODUCED VOLUNTAR- ILY BY THE RESPIRATORY MUSCLES

3.1 Material and method

Subjects

The experimental subjects were 12 young healthy students, mostly graduate students of medicine and physical education 8 males and 4 females, aged from 19 to 31. The experiments were carried out during autumn 1971 and spring 1972.

Measurement of respiratory volume

The experiments were carried out in a quiet room. The temperature was 20°C. During the registration the subjects were in a supine position. During the experiment all tight clothing had been either taken off or at least opened in order to give space for free movements of the thorax. Before the beginning of the experiment the subject rested for about 10 minutes.

A single session lasted from 45 to 90 minutes. To avoid possible hypocapnia in the blood, 5—10 quiet breathings separated the relevant breathings, and there were 1—2 pauses 5—10 minutes in duration. A mouth gag was placed into the subject's mouth and breathing was performed through it. In order to exclude breathing through the nose a nose clip was employed

as well. In order to avoid condensation of humidity the inspiratory air was warmed by electrical resistance. During the experiments 1872 inspirations and 2101 expirations were registered.

Respiratory volume which represents the mechanical activity of the respiratory muscles was recorded with a Fleischpneumotachographhead. An electric integrator transformed the flow into volume. The results were visualized on a four-channel Mingograph plotter from which the respiratory volumes were manually measured. After each experiment the volume was calibrated with a piston pump of known volume. The recording system is seen in Figure 1.

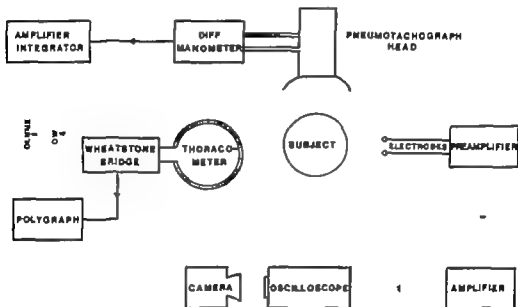


Fig 1 The recording system.

Psychophysical procedures

Three separate psychophysical classes of sensory scales were construed (See STEVENS 1959)

- 1 Category scale
- 2 Magnitude scale
- 3 Discriminability scale

To obtain category scale category production was used. In

Inspiratory experiments numbers were named by the experimenter in an irregular order from one to ten where zero represents the functional residual capacity (FRC) and ten the state of maximal inspiration. The subject was instructed to inspire volumes of air whose subjective magnitudes were equally spaced among the ten categories presented by the experimenter. Expiration was studied in a similar manner. The subjects always started from the FRC-state and then inspired maximally which was called the total lung capacity (TLC). The subject then expired in random order volumes between one and ten, with ten representing the maximal expiration.

Magnitude estimation was used to obtain a magnitude scale. The subject inspired from the FRC-state until the experimenter signaled that a sufficient volume had been achieved and then the subject estimated its apparent magnitude using numbers that seemed to him proportional to the magnitude.

The magnitude estimation of expiration was studied in the same manner so that the subject always started from the FRC-state and then inspired maximally holding his breath, the TLC state. Then the subject expired slowly until the experimenter signalled him to stop. Then the subject estimated the expired volume using numbers that seemed to him proportional to the magnitude.

In order to obtain discriminability scale a just noticeable difference was produced. In inspiration the subject inspired volumes, starting from the FRC-state that seemed to him just greater than the previous volume. After each volume the subject made five to ten quiet inspirations and then returned to the FRC-level inspiring a subjectively just greater volume than the previous one. In this way the subject continued up to the maximal volume.

The expiration was studied in the same manner except that the subject started from the TLC-state and expired just noticeably greater volumes.

3 2 Results

Category production

As category production does not yield any simple function in logarithmic coordinates (STEVENS 1959) data obtained with this method are presented in linear coordinates in the present study

Figure 2 shows the function obtained in the inspiration and expiration experiments performed on 12 subjects. The regression coefficient of the inspiratory function was 2.51 and the Pearson correlation coefficient was 0.77 ($p < 0.001$ $df = 9$). The

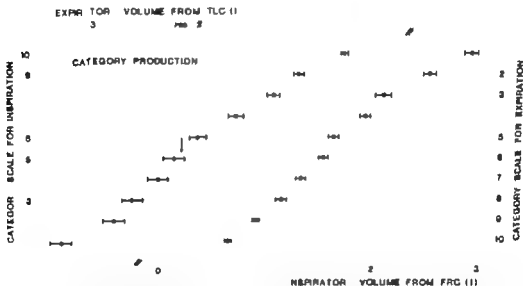


Fig 2 The subjective sensation on the category scale as a function of inspiratory (o) and expiratory (•) volume in a linear coordinate system. The points with ± 1 standard error of the mean are means of 10 subjects. The coefficient of linear regression in inspiration was 2.51 and in expiration 2.11. Notice the FRC level in expiration.

correlation coefficient indicates the interindividual consistency of estimation. The value of p was obtained from Fisher's statistical tables. The regression coefficient of the expiration function was 2.11 and the Pearson correlation coefficient 0.75 ($p <$

0.001 $df = 9$) On the ordinate the sensation quantity varies between one and ten. The respiratory volume is on the abscissa. The figure shows the means and the standard errors of the means (Interindividual variation $df = 9$ for inspiration and 9 for expiration) Figure 3 shows the results for each subject and the IntraIndividual variability of the means.

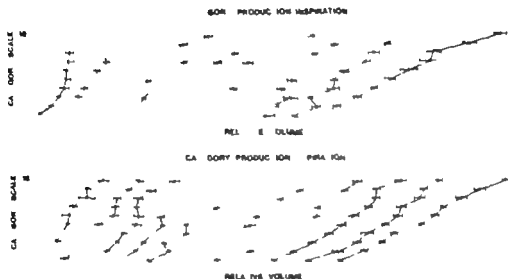


Fig 3. The results of individual subjects in a linear coordinate system. The individual regression coefficients and Pearson correlation coefficients are seen in the Appendix. The origin of the abscissa scale is arbitrary but the distance between each scale mark is one liter.

Magnitude estimation

Following STEVENS (1959) the results are presented in log log coordinates.

The results are shown in Figure 4 and appear to follow the power law. The magnitude is scaled from one to ten. The coefficient of log log regression (the value of the power law exponent) was 1.00 in inspiration and the Pearson correlation coefficient 0.57 ($p < 0.05$, $df = 9$). The corresponding regression coefficient of the expiration was found to be 1.03 and the Pear

son correlation coefficient 0.68 ($p < 0.01$ $df = 9$) The standard deviation of the individual regression coefficients was 0.417 ($n = 10$) in inspiration and 0.279 ($n = 10$) in expiration Figure 5 shows the results for each subject.

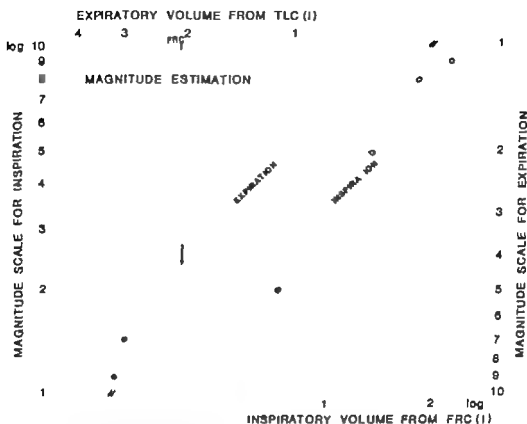


Fig 4. The subjective sensation as a function of inspiratory and expiratory volume in log-log coordinates. The magnitude scale is scaled from one to ten. The points are means of the results of 10 subjects.

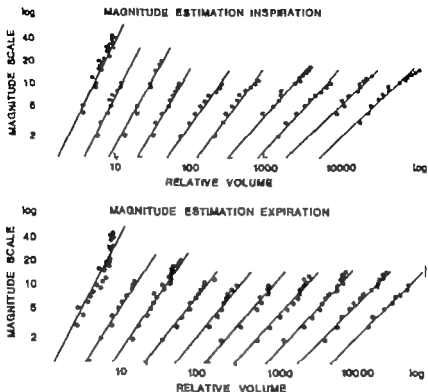


Fig 5 The individual results in a log log coordinate system. The lines have been displaced by integral factors to facilitate presentation. The individual regression coefficients and Pearson correlation coefficients are seen in the Appendix.

Discrimination

Following STEVENS (1959) the results are presented in a semilog coordinate system. The results are shown in Figure 6. Discrimination was scaled from one to ten. The results do not follow the Fechner law as can be seen from the curvature of the resulting functions. In inspiration the coefficient of the semilog regression was found to be 3.02 and the Pearson correlation coefficient 0.37 ($p < 0.05$ $df = 4$). In expiration the corresponding values were 2.93 and 0.70 ($p < 0.001$ $df = 4$). Figure 7 shows the results for each subject.

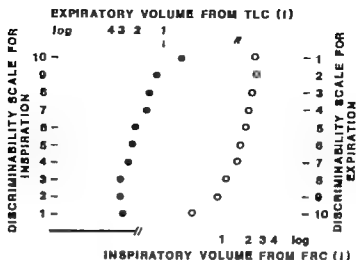


Fig 6. The subjective sensation on the discriminability scale as a function of inspiratory (o) and expiratory (●) volume in a semilog coordinate system. Discrimination is scaled from one to ten. The points are means of 10 subjects. The regression coefficient in inspiration was 3.02 and in expiration 2.93

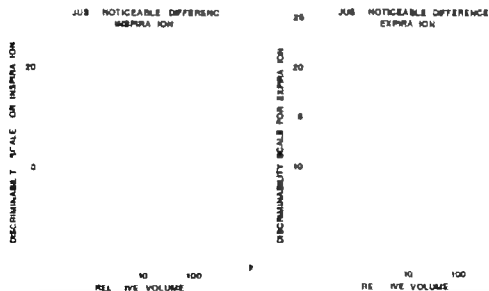


Fig 7 The individual results in semilog coordinate system. The subjects have been displaced by integral factors to facilitate presentation. The individual regression coefficients and Pearson correlations coefficients are seen in the Appendix

4. THE ELECTRICAL ACTIVITY OF HUMAN INTERCOSTAL MUSCLES AND SENSATION MAGNITUDE DURING VOLUNTARY INSPIRATION

4.1 Material and method

Subjects

The experimental subjects were 14 young, healthy male students of medicine and physical education aged from 20 to 24. Since the EMG registration was derived from surface electrodes subjects with a thick subcutis were excluded. During the experiments 665 inspirations were registered.

Registration of electrical activity

The electrical activity of the intercostal muscles was registered electromyographically. The recordings were carried out in a Faraday cage under quiet laboratory conditions. The electromyograms were registered with bipolar surface electrodes (VII. JANEN 1987). The electrode distance was 25 mm and the diameter of an electrode 7 mm. The ordinary electrodes were pieces of felt, clamped on plastic and fixed with Tensoplast to the skin, which had been cleaned with ether. Before the experiments, the felt was moistened with electrode fluid.

It can be assumed that an EMG activity sample obtained by surface electrodes represents the total electrical activity of the intercostal muscle (LIPPOLD 1952, BERGSTROM & KERTTULA

1961) The following measurements were calculated visually from the film. 1 The total number of EMG impulses (n number) during inspiration. 2. The EMG impulse frequency during the last 1/3 second of inspiration.

The EMG impulses were counted by two persons who did not know each others results, and a third checked the counting by arbitrarily selected samples. All impulses which were distinguishable from the baseline were counted as EMG impulses. The method was the same as that used by BERGSTRÖM (1958) and by VILJANEN (1967)

Psychophysical procedures

The subjective-multiple method that belongs to the category group was used in the experiments.

Before the beginning of the experiment the smallest volume at which EMG could be registered was sought. When it was found it was determined as the first volume on the subjective scale. Generally it varied between 0.5 and 0.75 liters. After this the subject was instructed to draw given subjective multiples of the base value. Each subjective multiple was registered ten times, the mean number of multiples being six. The number of multiples varied from 4 to 9 and the maximum corresponded to the maximal inspiration.

Registration of mechanical activity

Recording of the inspiratory volume and flow was performed on a Fleischpneumotachograph head, by means of an Elama Schönander differential manometer electrical integrator and a four-channel polygraph Mingograph 42. After each experiment the volume was calibrated with a piston pump of known volume

4.2. Results

The relationship between the voluntry sensation and the number of EMG impulses

As in section 3 the results are shown in linear coordinates Figure 8 shows the function obtained during inspiration experiments on all subjects. The subjective sensation is scaled on the ordinate one to ten and on the abscissa the total number of EMG impulses registered from intercostal muscles during inspiration. The figure shows the means and the standard errors of the means. The regression coefficient was found to be 0.01 and the Pearson correlation coefficient 0.61 ($p < 0.01$ $df = 13$)

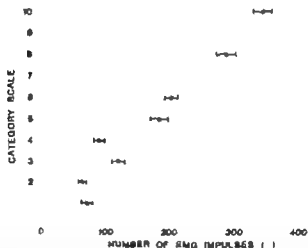


Fig 8. The subjective sensation as a function of the total number of electromyogram impulses during inspiration in a linear coordinate system. The points with ± 1 standard error of the mean are means of 14 subjects. The sensation is scaled from one to ten. The coefficient of linear regression was 0.01.

The relationship between voluntary sensation and the EMG impulse frequency

Figure 9 shows the relationship between the subjective sensation and the EMG frequency. The sensation is scaled from one

to ten. The regression coefficient was found to be 0.03 and the Pearson correlation coefficient 0.49 ($p < 0.05$ $df = 13$)

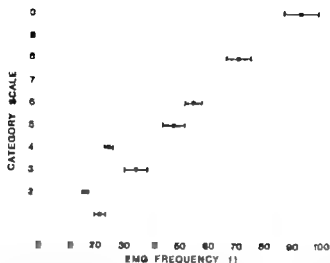


Fig 2. The subjective sensation as a function of electromyogram impulse frequency during inspiration in a linear coordinate system. The points with ± 1 standard error of the mean are means of 14 subjects. The coefficients of linear regression was 0.03.

5 THE INFLUENCE OF CARBON DIOXIDE STIMULATION ON THE RELATIONSHIP BETWEEN SENSATION MAGNITUDE AND VOLUME PRODUCED BY THE RESPIRATORY MUSCLES

5.1 Material and method

The experimental subjects were 8 healthy young students aged from 20 to 29. Six of the subjects had previously participated in physiological investigations performed by means of psychophysical methods, and two of them were novices.

The experimental procedure was the same as in the preceding study. The subjects breathed in through a flap valve from a plastic bag containing 2000 liters $5.0 \pm 0.5\%$ CO_2 air and expired through another valve into the atmospheric air.

The following psychophysical methods were used in the experiments.

1. Category production
2. Magnitude estimation

The course of the experiments corresponded with that of similar experiments performed in normal conditions. During the experiments 1578 inspirations and 1443 expirations were registered.

5.2 Results

Category production

During the CO_2 stimulation the regression coefficient of the inspiration function was found to be 2.65 in a linear coordinate system and the Pearson correlation coefficient 0.83 ($p < 0.001$ $df = 7$). The regression coefficient of the expiration function was found to be 2.01 and the Pearson correlation coefficient 0.84 ($p < 0.001$ $df = 8$). The results are shown in Figure 10. Figure 11 shows the results for each subject.

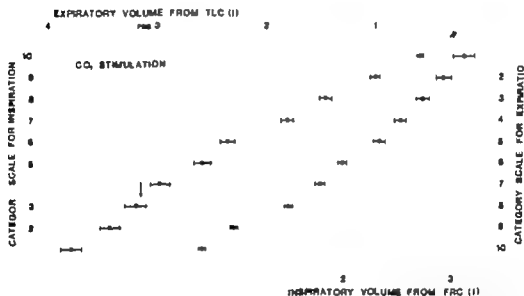


Fig 10 The subjective sensation on the category scale as a function of inspiratory (○) and expiratory (●) volume in a linear coordinate system during CO_2 -stimulation. The points with ± 1 standard error of the mean are means of 8 subjects. The coefficient of linear regression was in inspiration 2.65 and in expiration 2.01. Notice the FRC level in expiration.

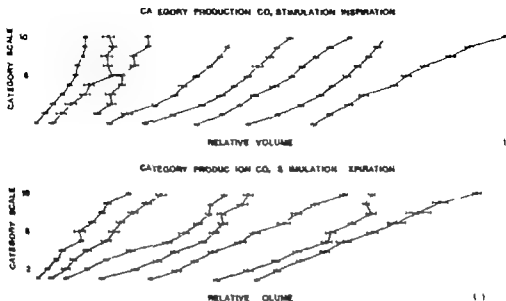


Fig 11. The individual results in a linear coordinate system during CO₂ stimulation. The individual Pearson correlation coefficients and regression coefficients are seen in the Appendix.

Magnitude estimation

The exponent of the power function (coefficient of the log log linear regression) obtained in the inspiration tests was 1.19 and the Pearson correlation coefficient 0.90 ($p < 0.001$ $df = 7$). In expiration tests the regression coefficient was 0.99 and the Pearson correlation coefficient 0.93 ($p < 0.001$ $df = 7$). In Figure 12

the results are seen in a log log coordinate system. The magnitude is scaled from one to ten. The standard deviation of the individual regression coefficient was in inspiration 0.307 ($n = 8$) and in expiration 0.128 ($n = 8$). In Figure 13 the individual results are shown.

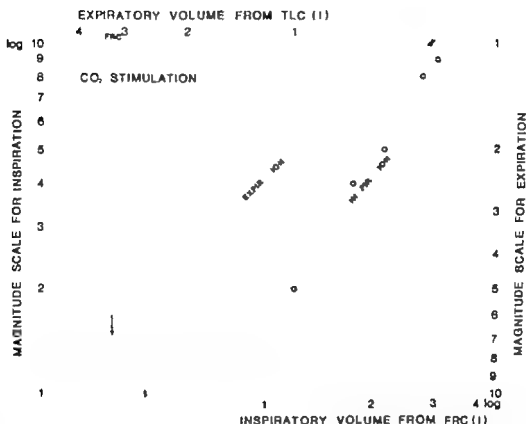


Fig 12. The subjective magnitude as a function of inspiratory and expiratory volume in log log coordinates during CO_2 -stimulation. The sensation is scaled from one to ten. The points are means of the results of 8 subjects.

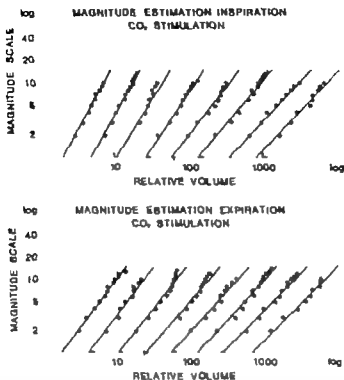


Fig 12. The individual results in a log-log coordinate system during CO₂ stimulation. The lines have been displaced by integral factors to facilitate the presentation. The individual regression coefficients and Pearson correlation coefficients are seen in the Appendix.

The comparison between normal conditions and CO₂ stimulation

On comparing the results obtained with the magnitude method both under normal conditions and during CO₂ stimulation it was found that the regression coefficient in inspiration during CO₂ stimulation became higher ($t = 3.573$ $df = 1591$ $p <$

0.001) and the cutting point of axis y lower ($t = 23.970$, $df = 1591$, $p < 0.001$) than under normal conditions. The differences are seen in Figure 14. It can be seen from the figure that a larger volume was inspired by the same subjective magnitude during CO_2 stimulation.

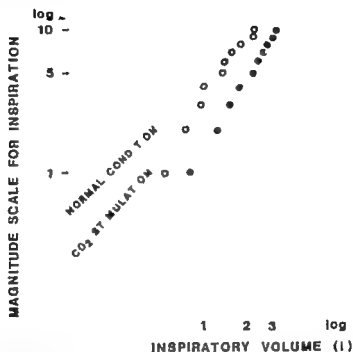


Fig 14. The effect of CO_2 stimulation on the relationship between the voluntary sensation and the inspiratory volume. The points are means of the results of 18 subjects

6 DISCUSSION

In attempts to clarify the voluntary control of the human respiratory muscles on a higher central level the greatest difficulty is measuring these control functions directly. When a psychophysical procedure is chosen as a method, the subjective sensation produced by the subjects' respiratory muscle movements can be used as a yardstick of the higher control which can be considered to represent the voluntary drive to the higher respiratory centers well. Earlier the psychophysical method had been found reliable in experiments made to explore the voluntary control of the human skeletal muscles (STEVENS & MACK 1959, EISLER 1962, BERGSTROM, HALTTUNEN & VILJANEN 1972).

In the present study scales of sensation were constructed by using the methods of just noticeable difference, category production and magnitude estimation.

Problem 1 Relationship between sensation and respiratory volume

In the present study a consistent relationship between the subjective sensation and the corresponding volume produced by the respiratory muscles was found. This followed the power law with the magnitude method.

In comparing the different methods used in the present study it can be found that the best correlated set of inspiratory and expiratory data was found with the category method. A clear inconvenience in carrying out the discriminability experiments was the fact that the experiments tended to become too long and obvious fatigue was detectable. Furthermore the scales formed by different subjects were quite different and thus less suitable for comparison. Perhaps for that reason, the overall function obtained did not follow the Fechner law

The fact that the category method in these experiments gave the best correlated results can be explained by the fact that in sensory physiological experiments in general the limits of the studied area are unknown to the subject but the respiratory movements present a clearly limited occurrence to the subject, whereby he knows the beginning and the termination points of the action. Under these circumstances the category scale may suit better for this kind of limited range in particular since it is known that the respiratory movements as such are a considerably more complicated process than normal muscular work, and therefore it is easier for the subject to operate by means of a given scale

In comparing the regression coefficients obtained in inspiration and expiration it is found that larger coefficients were obtained in inspiration than in expiration except with the magnitude method. This may suggest that inspiratory and expiratory muscles differ from each other in the amount of higher control, though both are also autonomically controlled. Further it can be noticed that the Pearson correlation coefficient in expiratory experiments was higher than in the inspiratory experiments (see Table 1) in other words, the voluntary control of the expiratory muscles would be more accurate (compare vocalization)

In the present experiments the exponent for inspiration was 1.00 and for expiration 1.03 obtained with magnitude estimation. It has been observed earlier that handgrip gives an exponent of 1.7 (STEVENS & MACK 1959) and the muscular force of the leg gave the same (EISLER 1962). It is interesting that the exponent values obtained are so similar in spite of the fact that the physical correlate in the present experiments is not force but a combination of force and work.

Problem 2. : Relationship between sensation and electrical activity

In the second part of the present study a clear relationship between subjective sensation and the electrical activity registered from the intercostal muscles during inspiration was also found in the linear coordinate system. In previous experiments on human respiratory muscles a clear relationship was found between the subjective sensation and the total number of EMG impulses registered from respiratory muscles, as well as between sensation and the EMG frequency (VILJANEN 1970 1972) in the present study which used a larger material, it was found that sensation has a higher correlation coefficient to the total number of EMG impulses than to their frequency (see Table 1) BERGSTROM (1957 1958) arrived at the same results in his experiments on the skeletal muscles.

Problem 3 CO₂ stimulation

In the third part of the present study the aim was to investigate how the increased CO₂ stimulation would affect the relationship between subjective sensation and the volume produced by the respiratory muscles. It was found that with both methods a higher correlation coefficient was achieved with CO₂, than during normal conditions (see Table 1) Also the inspiratory regression coefficient was found to be higher and the cutting point of axis y lower than during normal conditions. This means that a greater volume was achieved with the same sensation during the CO₂ stimulation.

It has been observed earlier that the same respiratory muscle force is achieved with a smaller number of EMG impulses during CO₂ breathing (VILJANEN 1967) Thus, this kind of CO₂ effect, in which a greater volume equals the same sensation quantity may be closest to a direct involuntary autonomic effect. The results also agree with the observations made in experiments on voluntary muscles (BERGSTROM 1957 1958) and

sensory physiological experiments (JARVILEHTO 1973) where sensation has been found to follow the electrical activity better than the mechanical activity. Furthermore, it has been verified in earlier experiments that the final state of inspiration is determined and after starting the inspiration the impulse pattern no longer changes (VILJANEN 1967, 1970).

It may be concluded that the voluntary action controls respiratory functions in the same way as it does the other voluntary muscles. The vegetative centers control the respiratory muscles to get an adequate interchange of gases which in turn does not function as a control of the subjective will.

Voluntary control may regulate respiratory movements only as far as these are in the same position as ordinary motor movements, like among other things speech, singing, eating, diving etc.

The results obtained may be of interest in investigating the interaction between conscious central and autonomic functions.

7 SUMMARY

1. In the present study comparisons were made by means of a psychophysical method between the subjective sensation and respiratory volumes produced by the respiratory muscles. Further the relationship between sensation and EMG activity registered from the intercostal muscles were studied during inspiration. The effect of CO_2 stimulation on the relationship between sensation and volume was also observed

2. The subjective sensation followed the power law with the method of magnitude estimation.

3. The sensation was found to have a higher correlation to total number of EMG impulses than to EMG frequency

4. During CO_2 stimulation a greater respiratory volume equalled the same sensation magnitude

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APPENDIX

TABLE 1 Numerical values of linear regression coefficients and Pearson correlation coefficients in different experimental groups and with different transformations of the coordinates.

	Category production	Magnitude estimation	Discontinuation	Number of EMG impulses	Frequency	Category production	Magnitude estimation
	normal condition				CO ₂ stimulation		
Inspiration							
$y = bx + a$	$y = 2.51x + 1.39$			$y = 0.01x + 3.45$	$y = 0.02x + 4.07$	$y = 2.45x + 0.85$	
Pearson correlation coefficients (lin/lin)	0.77			0.81	0.49	0.83	
$y = b \log x + a$							
Pearson correlation coefficients (lin/log)			$y = 2.02 \log x + 4.85$				
			0.37				
$\log y = b \log x + a$		$\log y = 1.00 \log x + 1.33$					$\log y = 1.18 \log x + 0.73$
Pearson correlation coefficients (log/log)		0.57					0.90
Expiration							
$y = bx + a$	$y = 2.11x + 1.16$					$y = 2.01x + 0.81$	
	0.75					0.84	
$y = b \log x + a$			$y = 2.60 \log x + 3.17$				
			0.70				
$\log y = b \log x + a$		$\log y = 1.00 \log x + 0.74$					$\log y = 0.89 \log x + 0.79$
							0.81

TABLE 2 Numerical values of individual regression coefficients and Pearson correlation coefficients in different experimental groups

CATEGORY PRODUCTION

Inspiration

subject and experiment code	scale	number of inspirations and expl rations	regression coefficient in linear coordinates	correlation coefficient in linear coordinates	regression coefficient in log-log coordinates	correlation coefficient in log-log coordinates
43	1-10	49	3.18	0.79	1.25	0.79
44	1-10	80	2.94	0.97	1.27	0.98
46	1-10	100	2.11	0.94	1.07	0.94
52	1-10	100	3.79	0.96	1.42	0.96
53	1-10	55	3.53	0.97	1.34	0.96
54	1-10	80	3.62	0.96	1.22	0.95
55	1-10	80	3.00	0.72	0.96	0.75
61	1-10	80	5.19	0.85	1.87	0.88
62	1-10	100	6.44	0.94	1.46	0.96
63	1-10	100	8.46	0.63	1.58	0.93

Expiration

42	1-10	44	3.82	0.84	1.66	0.83
45	1-10	50	2.57	0.96	1.17	0.94
47	1-10	51	4.19	0.96	1.39	0.95
48	1-10	100	6.14	0.94	1.42	0.95
49	1-10	91	4.36	0.79	1.36	0.87
50	1-10	100	2.99	0.94	1.42	0.97
51	1-10	100	2.41	0.97	1.34	0.96
60	1-10	30	1.72	0.94	0.72	0.93
64	1-10	44	2.78	0.93	1.45	0.96
71	1-10	187	2.41	0.82	1.11	0.85

MAGNITUDE ESTIMATION

Inspiration

1	1-10	98	3.66	0.96	1.34	0.94
2	1-45	110	36.66	0.92	1.06	0.91
3	1-6	60	3.62	0.92	1.07	0.90
5	1-8	80	8.23	0.85	1.66	0.86
7	1-8	62	4.03	0.87	1.31	0.89
9	1-10	83	5.06	0.86	0.95	0.86
11	1-12	80	4.45	0.94	1.10	0.94
14	1-10	100	5.37	0.93	1.66	0.94
15	1-12	86	5.21	0.92	0.95	0.92
16	1-20	59	9.01	0.91	1.76	0.92

Expiration

17	2-45	104	23.10	0.80	1.67	0.86
18	1-10	99	2.94	0.86	1.21	0.89
19	1-15	99	4.38	0.96	1.06	0.96
20	1-20	100	6.61	0.92	1.45	0.92
21	1-15	92	3.77	0.95	1.00	0.96
22	1-15	101	2.69	0.96	1.00	0.96
23	1-13	97	3.39	0.89	1.06	0.95
25	1-11	97	3.11	0.94	1.63	0.95
170	1-14	100	2.65	0.96	1.13	0.96
172	1-12	100	2.77	0.92	1.25	0.95

JND

subject and experiment code	scale	number of inspirations and expl rations	Inspiration			correlation coefficient in semilog coordinates	correlation coefficient in semilog coordinates
			regression coefficient in linear coordinates	correlation coefficient in linear coordinates	regression coefficient in semilog coordinates		
31	1-15	60	4.45	0.94	6.54	0.98	0.98
32	1-16	64	4.07	0.95	4.93	0.91	0.91
33	1-21	84	17.59	0.90	11.88	0.91	0.91
35	1-15	80	4.99	0.96	6.34	0.90	0.90
36	1-21	82	11.96	0.94	10.39	0.90	0.90

Expiration

30	1-21	82	6.11	0.93	6.51	0.91	0.91
38	1-17	65	3.23	0.97	5.32	0.91	0.91
39	1-15	58	3.78	0.93	6.82	0.87	0.87
40	1-16	62	3.75	0.94	6.25	0.87	0.87
56	1-21	79	6.94	0.94	10.08	0.90	0.90

TABLE 2. Numerical values of individual regression coefficients and Pearson correlation coefficients in EMG registration

CATEGORY PRODUCTION									
EMG									
subject code	scale	number of inspiration	frequency				number of EMG impulses		
			regression coefficient in linear coordinates	correlation coefficient in linear coordinates	regression coefficient in log-log coordinates	correlation coefficient in log-log coordinates	regression coefficient in linear coordinates	correlation coefficient in linear coordinates	regression coefficient in log-log coordinates
1	1-6	30	0.12	0.75	1.40	0.79	0.020	0.62	1.51
2	1-6	56	0.02	0.74	0.49	0.86	0.008	0.72	0.51
3	1-6	59	0.02	0.89	0.69	0.77	0.007	0.86	1.02
4	1-5	25	0.18	0.87	2.15	0.89	0.017	0.89	1.41
5	1-5	49	0.07	0.89	1.04	0.82	0.013	0.90	0.85
6	1-6	80	0.01	0.77	0.42	0.81	0.005	0.84	0.38
7	1-6	30	0.04	0.77	0.21	0.54	0.010	0.78	0.75
8	1-6	57	0.08	0.94	0.86	0.95	0.009	0.88	0.84
9	1-6	83	0.05	0.83	0.94	0.83	0.010	0.91	0.81
10	1-6	56	0.07	0.80	0.48	0.79	0.026	0.74	0.48
11	1-9	43	0.12	0.74	1.18	0.68	0.022	0.78	0.97
12	1-6	50	0.07	0.77	1.03	0.79	0.023	0.77	0.87
13	1-4	40	0.06	0.83	0.83	0.81	0.017	0.88	0.81
14	1-5	49	0.07	0.84	0.76	0.83	0.019	0.81	0.71
									0.86
									0.87
									0.88
									0.89
									0.92
									0.95
									0.60
									0.85
									0.82
									0.76
									0.85
									0.77
									0.83
									0.80

FATE OF NORADRENALINE
IN THE HUMAN FOETOPLACENTAL UNIT

IN VIVO STUDIES ON PLACENTAL TRANSFER,
METABOLISM AND DISTRIBUTION IN
FOETAL TISSUES

BY

SEPPO SAARIKOSKI

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 421

FROM THE DEPARTMENT I OF OBSTETRICS AND GYNAECOLOGY UNIVERSITY
OF HELSINKI MEDICAL SCHOOL, HELSINKI, FINLAND

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ABBREVIATIONS

A	= Adrenaline
CA	= Catecholamine
COMT	= Catechol -O- methyl transferase
CR	= Crown rump length
DA	= Dopamine
DHMA	= 3,4-dihydroxymandelic acid
DHPG	= 3,4-dihydroxyphenylglycol
DOPA	= 3,4-dihydroxyphenylalanine
DOPAC	= 3,4-dihydroxyphenylacetic acid
HVA	= Homovanillic acid
MNO	= Monoamine oxidase
MHPG	= 3-methoxy-4-hydroxyphenylglycol
ME	= Metanephrine
MT	= 3-methoxy tyramine
NA	= Noradrenaline
NME	= Normetanephrine
VMA	= Vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid, MOMA)
VA	= Vanillic acid

INTRODUCTION

The adrenergic nervous system plays a central role in the regulation of the foetal circulation under normal conditions and in foetal adaptation to stress, e.g. to asphyxia, as well as, finally in the adaptation of the newborn to extrauterine life (Dawes 1968, Rudolph and Heymann 1973)

High NA levels in the maternal peripheral blood are considered harmful for the foetus since they may increase uterine contractions and contract the uterine blood vessels, and thus impair the supply of blood to the foetus (Dornhorst and Young 1952, Beard 1962). This phenomenon is believed to be, at least partly responsible for the high perinatal mortality of children of pheochromocytoma patients (Blair 1963, Thiery Derom van Kets, de Schaepdryver Bernard, Beckaert, Hooft, Derom, Rolij and Roels 1967, Batts, Tchilinguirian and Passmore 1974). Similarly those CAs which cross the foetoplacental barrier from the maternal circulation may interfere with the shunts regulating the foetal circulation and could possibly impair the adaptation of the foetus to a crisis situation (Owman, Aronson, Gennser and Sjöberg 1973).

Our knowledge of the functioning of the adrenergic nervous system in the human foetus is based mainly on *in vitro* studies, on determinations of the CA content of foetal tissues, histological and electron microscopic studies and demonstrations of enzyme and receptor activities. The dynamic behaviour of the mediator substance of the adrenergic nervous system, NA, and its metabolites in the maternal placental foetal system is *also* is, however poorly understood. Such an understanding is essential if the role of the adrenergic nervous system of the human foetus under normal conditions and in those deviating from normal is to be completely elucidated.

REVIEW OF THE LITERATURE

Passage of noradrenaline through the placenta, and its metabolism in the placenta

Placental transfer It has been shown that radioactive NA can traverse the placenta of sheep and guinea-pigs to some extent (Condorelli and Cosmi 1970 Cosmi and Condorelli 1973, Morgan, Sandler and Panigel 1972) but not that of rats or dogs (Mirkin, unpublished data 1971 ref. Mirkin 1973). The passage of NA across the human placenta was thought possible after intravenous administration of CA to mothers, on the basis of indirect indications gained by monitoring foetal heartbeat or foetal glucose and free fatty acid levels (Beard 1962, Zurpan, Whaley Nelson and Ahlquist 1966) but reports contradicting these results have also appeared (Pardi, Tiengo, Uderzo, Salmoiraghi and Candiani 1969).

After injection into mothers, ^{14}C -NA and ^{14}C -A have been shown to pass into unviable foetuses in demonstrable quantities, within 2 to 5 min. The ^{14}C -NA in the umbilical blood of two foetuses in the second trimester was 2% and 18% respectively of the maternal plasma concentration (Sandler Ruthven, Contractor Wood, Booth and Pinkerton 1963 Sandler Ruthven and Wood 1964).

Metabolism in the placenta. NA metabolism in the placenta is catalyzed both by the action of MAO and COMT (Pesci and Camone 1947 Luschnicky and Singher 1948, Thompson and Tickner 1949 Isalo and Castrén 1967 Klinge 1968). The very high placental MAO activity has been observed to decrease towards the end of the pregnancy with decreasing partial oxygen pressure, and also in toxæmia (Sandler and Covey 1962 de Maria 1964 Nowick 1968, Sagone and Arrotta 1966). In toxæmia, the MAO activity was not considered lowered unless the placenta was degenerated (Klinge, Penitile and Tisari 1964 Castrén and Saarikoski 1969).

In vivo in placental homogenates and during placental perfusion, NA is metabolized mainly by the action of MAO (Castrén, Saarinen and Saarikoski 1968, Morgan *et al.* 1972 Castrén and Saarikoski 1974). During transplacental passage in guinea-pig and in man, the *in vivo* metabolism also appears to be mediated through both MAO and COMT action (Morgan *et al.* 1972, Saarikoski 1972).

The NA concentration in the umbilical blood of children of pheochromocytoma patients is only about 10% of the maternal level. The VMA in the umbilical blood of one foetus was five times higher than in the mother's peripheral blood. This would

also suggest that NA is metabolized during its transplacental passage (Barzel, Bar Ilan, Rumney, Lazebnik, Eckerling and de Vries 1964; Beckert, Barber van Germeersch and Thiery 1966).

There is no information available on the conjugation of NA and its metabolites in the placenta. Conjugation is unlikely since no p-nitrophenol-sulphatase or o-aminophenol-conjugating UDPG-hydrogenase or glucuronyl transferase activity is found in the placenta, (Harttala and Pulkkinen 1955, Dutton 1959, Pulkkinen 1963).

Noradrenaline in foetal tissues

In order to assess the functional status of the adrenergic nervous system in the foetus, it is necessary to estimate the foetus' ability to synthesize, store, inactivate and release transmitter substances from the nerve endings.

Peripheral tissues. The CA content of many adrenergically innervated tissues, such as the heart, is low in chicken embryo (Ignarro and Shudeman 1968a) and in the foetuses of several mammals (Glowinski, Axelrod, Kopin and Wurtman 1964; Iversen, de Champlain, Glowinski and Axelrod 1967; Friedman, Pool, Jacobowitz, Seagren and Braunwald 1968; Lipp and Rudolph 1972). The CA content of peripheral foetal tissues conforms to the ontogenetic principle that in those foetuses which are well developed at birth there is a higher content than in those which are less well-developed.

In human foetal heart no adrenergic innervation is histochemically demonstrable before the twelfth week of gestation, or in the bowel before the ninth to tenth week (Gensser and Nilsson 1970; Read and Burnstock 1970). As early as the second trimester, however, the NA content of the heart and many peripheral tissues in the human foetus reaches levels similar to those found in adult tissues of many species (Greenberg and Lind 1961). Using histochemical procedures the human foetal circulatory shunts have been observed to be adrenergically innervated (Bortus, Malmfors, McMurphy and Olson 1969; Aronson, Gensser, Owman and Sjöberg 1970). The adrenergic innervation of the umbilical cord, observable by fluorescence histochemistry, extends only about 1 cm outside the body (Bortus *et al.* 1969).

Brain. The NA content in the foetal brain also appears to conform to the ontogenetic principle. In rat brain the NA content three days before birth is only 15% of the adult level, while in the guinea-pig, which is considerably more mature at birth, cerebral NA concentrations have already attained adult levels at this time (Härki, Kuntzman and Brodie 1962).

The cerebral NA content is low in human foetuses during the first half of pregnancy (Bertler 1961; Greenberg and Lind 1961; Hyypä 1972). CA fluorescence was observable in the human foetal hypothalamus from the tenth week of pregnancy and in the median eminence from the 13th week. The DA concentration in the hypothalamus is already clearly higher than the NA concentration during the 11th to 15th weeks of pregnancy (Hyypä 1972). The topography of the monoaminergic paths in the brain of the human foetus during the second trimester is in its main parts consistent with that observed in the rat 1–2 weeks after birth (Nobun and Björklund 1973).

Chromaffin tissue The chromaffin tissue in the foetus matures at an earlier stage than the adrenergic postganglionic nerves. Primitive sympathetic cells may already be found in human foetuses of 14–16 mm length, and pheochromocytes proper in foetuses 27–46 mm long (Coupland 1952). According to Hervonen (1971) differentiation of primitive sympathetic cells in human foetal aortic paraganglia (Zuckerlandl 1901) and adrenal medulla occurs during the eighth week of gestation.

During the foetal period, the adrenal medulla and sympathetic paraganglia carry the greater part of the total CA content of the foetus and a relatively greater proportion than in adult life (Hökfelt 1951, Shepherd and West 1951, 1952, Comline and Silver 1966). The NA concentration in the aortic paraganglia is higher than in the adrenals (Niemiöva and Pekkarinen 1952, Coupland 1953).

Towards the end of the intrauterine period the A/NA ratio increases in the adrenal medulla. The adrenal cortical hormones, hydrocortisone in particular, are thought to regulate the A synthesis (Roffi 1965, Mangolia, Roffi and Jost 1966, Wurtman and Axelrod 1966). The human foetal adrenal medulla is capable of methylating NA to A already during the first half of gestation (Niemiöva and Pekkarinen 1952, Greenberg and Lind 1961, v Staudnitz 1968).

The sympathetic paraganglia, on the other hand, contain NA, almost exclusively throughout the foetal period (Greenberg and Lind 1961, Brundin 1965, Gensser and v Staudnitz 1969b).

Metabolism of noradrenaline in the foetus

The metabolic inactivation of CA is achieved mainly through the action of MAO and COMT and conjugating systems (for ref., see Axelrod 1965, Iversen 1967, Sharman 1973).

MAO In 1928 Hare described an enzyme which oxidized tyramine. It was later found that this enzyme was also responsible for the decomposition of A and NA in rat, guinea-pig and rabbit liver (Blaschko, Richter and Schlossmann 1937a). MAO is present in most tissues but it is particularly abundant in the liver, intestine, kidneys and central nervous system (Blaschko, Richter and Schlossmann 1937b). The MAO distribution in the human differs from that in many animals in that human heart displays high MAO activity compared to the lower levels seen in many other species (Langemann 1944, Levine and Sjoerdama 1962).

Many foetal tissues display low MAO activity. In the chicken embryo MAO activity is measurable from the fourth day of gestation with the highest levels being obtained on the 19th day (Ignarro and Shideman 1968b). The MAO activity in rat foetuses during the foetal and early neonatal period is only about 20 % of adult values (Glowinski *et al.* 1964, Robinson 1967). The MAO activity in rabbit liver as late as two weeks postnatally only amounts to about 10 % of the adult level (Bojanek, Bozkowa and Kurzepa 1966). On the 25th day of pregnancy the MAO activity in guinea-pig kidney, liver and intestine is only 10 to 20 % while that in brain is already some 60 % of adult values. The hepatic and intestinal MAO activities subsequently increase rapidly but that of the kidneys increases more slowly. At birth, however, the MAO activity in all the tissues mentioned is about 60 % of the corresponding adult value (Timari 1966).

The human foetal MAO activity has also been considered low compared with that of the adult. The foetal hepatic MAO activity during the first to second trimesters is about 1/4 that of term placenta, which is known to be of the same order as the hepatic MAO activity (Luschnicky and Singher 1948, Davison 1958, Castrén and Saarikoski 1969). In homogenates and mitochondria of the human foetal liver cells MAO activity is constant from the 13th to 28th week of gestation and doubles during weeks 28 to 40 of development (Skorobohata and Drel 1973). In the aortic bodies and in the ductus venosus higher MAO activities than in the liver have been recorded, and in the umbilical blood vessels equal or lower activities (Gennær and v Studnitz 1969a, 1969b). In human foetal brain MAO activity is demonstrable from the 10th to the 12th week of pregnancy onwards (Himberg and Solatunluri, personal communication 1973).

COMT COMT is an enzyme responsible for 3-O-methylation of NA and other catechols (Axelrod 1957). It catalyzes the transfer of a methyl group from S-adenosylmethionine to meta-hydroxyl groups in these substances. COMT occurs in a number of tissues, in particular in the liver and kidneys (Axelrod and Tomchick 1958, Axelrod, Albers and Clemente 1959).

The COMT activity of foetal tissues develops in a fashion similar to MAO activity: in the chicken embryo it is observable from the fourth day of incubation. (Ignarro and Shademan 1968b). The COMT activity of the newborn rat is about 20 % of the adult value (Glowinski *et al.* 1964).

In the human foetal heart, adrenals, liver and brain *in vitro* NA metabolism has been found to be mediated mainly by action of COMT. A similar situation also prevails *in vivo* in a number of foetal tissues during the second trimester (Castrén *et al.* 1968, Castrén and Saarikoski 1969, Saarikoski and Castrén 1971). In the foetal umbilical blood vessels or in the aortic bodies no COMT activity is demonstrable at the 12th week of gestation while in the liver the activity is already fairly high (Gennær and v Studnitz 1969a, 1969b).

On the basis of evidence obtained from post-mortem specimens it is considered that the COMT activity increases with increasing age: in a second trimester foetus the activity is about 13 % of that in the neonate and about 13 % of the activity in a 50-year-old person (Agathopoulos, Nicolopoulos, Mavaniotis and Papadatos 1971). Newborn excrete mainly O-methylated catecholamines and VMA in their urine in amounts which increase during the first days after birth but more distinctly in premature than in full-term infants. This situation was considered to indicate a deficiency in oxidative deamination activity at birth and, on the other hand increasing COMT activity during the immediate neonatal period (Brunjes, Castner and Hodgman 1964, Nicolopoulos, Agathopoulos, Dancilathou-Athanasiadou and Bafataki 1968).

Conjugation with glucuronic or sulphuric acid. Some A, NA, and CA metabolites are excreted in urine conjugated with glucuronic or sulphuric acid (Richter 1940 v Euler, Hellner, Björkman and Orwén 1955, Elmadjian, Lamson and Neri 1956, La Brosse, Axelrod, Kopin and Kety 1961, Haggendal 1963b, Goodall, Alton and Rosen 1964). The liver and intestine are thought to be the most important sites of conjugation, but conjugation also takes place in other tissues. The principal metabolite found in rat brain after intracisternal administration of ³H-labelled NA and NMN

was MHPG-sulphate (Schanberg, Schildkraut, Breese and Kopin 1968). In human spinal fluid MPHG was also present as its sulphate conjugate (Schanberg, Breese, Schildkraut, Gordon and Kopin 1968).

There is no positive indication of CA conjugation in the human foetus. Conjugation appears possible however since after H NA administration to the mother nearly 1/3 of the metabolites in the foetal liver were conjugates, conjugated NA, NMN and MHPG-VMA were detected (Saarikoski 1972). In the liver and other tissues of rabbit and human foetuses the activities of the glucuronidating enzyme system is low towards both p-aminophenol and steroids (Hartala and Pulkkinen 1955, Dutton 1959). In contrast p-nitrophenol, phenol and steroid sulphate ester formation has been demonstrated in human foetal liver, lungs, adrenals, kidneys and intestine (Pulkkinen 1963, 1966, Wengle 1964).

Uptake of noradrenaline and receptor function in the foetus

Uptake. The machinery required for the storage and binding of endogenously synthesized and exogenously administered CA appears to be poorly developed during the foetal period. Chicken embryonic heart can bind H NA from the fifth day that is from the time adrenergic innervation is histochemically detectable (Ignarro and Shadenman 1968c). H NA binding in the rat heart and spleen increases significantly only 2 to 3 weeks after birth, whereas intestinal and salivary gland NA binding capacity is the same at birth as in the adult (Glowinski *et al.* 1964, Iversen *et al.* 1967).

Human foetal intestine can bind NA *in vitro* before innervation can be visualized by fluorescence histochemistry (Read and Burnstock 1970). In the intact human foeto-placental unit *in vivo* H NA uptake by the foetal tissues appears comparatively poor (Saarikoski and Castrén 1971).

Receptor function. According to classical receptor theory two kinds of adrenergic receptors exist: adrenergic alpha receptors, stimulation of which usually produces an excitatory response in the cell, while stimulation of beta receptors most often results in an inhibitory response (Ahlquist 1948).

Frequent attempts have been made to study the function of the foetal adrenergic nervous system by determining the effect of adrenergic alpha and beta agonists and antagonists in various foetal tissues. The adrenergic receptors are believed to function at an early stage of foetal development, in animal as well as human foetuses. NA is thought to increase the cardiac frequency of the rat foetus *in vitro* prior to the 16th postcoital day but not after that, while A has a similar effect after but not before the 16th day (Bernard and Gargouil 1967).

In the human foetal heart, *in vitro*, A causes a chronotropic and ionotropic response already in the 9th or 10th week of pregnancy that is before the adrenergic innervation of the heart is histochemically demonstrable (Gennser and Nibbel 1970). In addition, from the 12th week the foetal cardiac response to A is comparable to that of the adult (Baker 1953). In the human foetal small intestine beta receptor function is clearly demonstrable *in vitro* from the 11th to the 23rd week of pregnancy (McMurphy and Boréus 1968). According to other authors, at this time alpha receptor function is also observable in human foetal small intestine (Hart and Mir 1971a) as well as

in the colon, where stimulation of alpha-adrenergic receptors causes contraction and that of beta-adrenergic receptors relaxation (Hart and Mir 1971b)

In the human foetus adrenergic receptor function is also demonstrable in the ductus arteriosus during *in vitro* perfusion and using the spiral strip experimental arrangement (Boréus *et al.* 1969). The results suggest that adrenergic mechanisms participate in the closure of the ductus arteriosus. NA, acetylcholine and serotonin also participate in the contraction of the intra-abdominal part of the umbilical vein and the initial part of the ductus venosus in the human foetuses of 20th to 24th weeks. A NA-like response can also be elicited with tyramine and can be inhibited with phenoxybenzamine, which blocks the alpha-adrenergic receptors. These results support the theory which proposes that a functional adrenergic sphincter mechanism exists at these points (Ebinger Gennar Owman, Pernon and Sjöberg 1968 Owman *et al.* 1973)

Catecholamines and foetal haemodynamics under normal conditions and in asphyxia

Normal situation. The greater part of our knowledge of foetal haemodynamics is based on animal experiments either with exteriorized foetuses or with so-called chronic preparations. Although activity of the adrenergic receptors of the end organs is demonstrable at an early stage of foetal development, rather little investigation of the contribution of the adrenergic nervous system to the regulation of the vasomotor tonus of the blood vessels, the cardiac frequency and the contraction force of the heart in the foetus has been made.

A injected into the rat foetus causes an increase in blood pressure and pulse pressure and bradycardia, injection into the mother results in lowered foetal blood pressure and pulse pressure and bradycardia (Girard 1965). NA and A injected directly into the foetal circulation of the sheep towards the end of pregnancy increase the contraction force of the heart, the carotid circulation and the blood pressure, from which ensues reflexory bradycardia (Pardi *et al.* 1969). According to the same authors the sensitivity of the foetus to these CAs is less than that of the adult, and it is lower at the beginning than at the end of the foetal period.

In sheep foetuses prepared for chronic experiments at a gestational age of 85 to 150 days, atropine and the alpha receptor-blocking agents phenoxybenzamine and phentolamine raise the heart frequency of the foetus even in the case of younger foetuses, which shows that both cholinergic and adrenergic regulation are active in the foetus (Vapaavuori Shirebourne Williams, Heymann and Rudolph 1973).

Using an *in utero* preparation and the microsphere and antipyrine method for studying the circulation and its distribution (Rudolph and Heymann 1967a, 1967b) beta adrenergic receptor stimulation with isoprenaline was found to increase the heart frequency, cardiac output, umbilical circulation and coronary circulation in the sheep foetus. On the other hand, there was no change in the proportion of the cardiac output received by other organs, nor did the blood pressure increase. In contrast, alpha-adrenergic receptor stimulation with methoxamine increased the blood pressure of the foetus and slightly reduced the cardiac output, the umbilical circulation, myocardial circulation and renal circulation (Rudolph 1969 Barrett, Heymann and Rudolph 1972, Rudolph and Heymann 1973).

The cortical activity of the brain also appears to influence the circulation of the sheep foetus *in utero*. The change in the EEG (brain curve) from the slow high voltage curve to the fast low voltage curve, which occurs during REM (rapid eye movement) sleep, is associated with a simultaneous irregular respiratory activity and an increase in the heart frequency and blood pressure (Dawes, Fox, Leduc, Liggins and Richards 1972).

It is not possible to perform similar chronic experiments with human foetuses: as a rule, all experiments that have been carried out were acute and asphyxia was a remarkable factor in them. NA causes an elevation of the blood pressure and increased heart frequency in the exteriorized second trimester human foetus (Enbörning and Westin 1954). Close to the end of pregnancy the human foetal heart appears to function with the same autonomous regulatory mechanisms as that of the adult (Renou, Newman and Wood 1969).

Asphyxic situation. The cardiovascular response to hypoxia appears to be associated with the functioning of the autonomous nervous system. In the sheep foetus an increase in systemic blood pressure, vasoconstriction and an increase in heart frequency in response to hypoxia are seen in 70 to 80 day of foetuses, that is at the time when the first response is elicited by stimulating the vagus nerve or the adrenergic nerves (Born, Dawes and Afott 1956, Dawes 1968).

In the newborn rabbit severe asphyxia results in a considerable depletion of the CA content of the extra-adrenal chromaffin tissue (Brundin 1966). In the human foetus, asphyxia of 10 to 15 min duration causes a significant decrease in CA fluorescence in the aortic bodies, but only a slight change in the CA fluorescence of the adrenal medulla (Hervonen and Korkala 1972). It has been suggested that during the foetal period functioning secretory innervation is probably lacking in the extra-adrenal chromaffin tissue. Thus NA would be liberated from this tissue primarily under the influence of chemical and humoral stimulation in response to asphyxia, wherein this tissue is thought to be of considerable significance (Lempinen 1964, Brundin 1966, Hervonen 1971, Hervonen and Korkala 1972).

Both sheep and bovine foetal adrenal medulla also contains cells which respond directly to asphyxic situations by releasing NA. These cells are sensitive to decreases in pO_2 , but not to increased pCO_2 ; lowering of the pO_2 to 4–5 mmHg causes NA release. Bovine foetal adrenal gland appears to contain only cells of this type. The innervated part of the sheep foetal adrenal medulla reacts in a similar manner to decreases in pO_2 . NA secretion increases when the pO_2 goes down to 12–16 mmHg and that of A when the pO_2 decreases to 8–12 mmHg (Comline and Silver 1961, 1966, Comline, Silver and Silver 1965). Closure of the umbilical cord causes increased foetal blood pressure; the beta-adrenergic receptor blockers react to prevent this increase, and this results in circulatory collapse (Joelsson and Barton 1969).

If the CA stores in a guinea-pig mother and foetus are completely emptied by administering reserpine, the incidence of perinatal mortality increases. Under such conditions the newborn cannot endure the postnatal asphyxia (Towell, Hyman, James, Steinland, Gerst and Adamsons 1965). Brief exposure to reserpine does not impair the response of newborn guinea pigs to asphyxia (Towell 1971). An intact functioning autonomous nervous system appears to be indispensable for the foetus successful adaptation to and management of asphyxia.

The foetal heart tolerates anoxia considerably better than that of the adult, and under *in vivo* conditions the human foetal heart is superior to that of many other species in this respect (Penn 1970). Adrenaline impairs the recovery of the foetal atrium from anoxia. The beta-adrenergic receptor blocking agent propranolol inhibits the effect of adrenaline (Penn 1970).

Effect of externalization. Some 40 to 50 % of the sheep foetal cardiac output goes to the placental circulation. The proportion received by the placenta from the human foetus is thought to be of the same order. When the foetus is removed from the womb the placental circulation rapidly abates. Within 30 min the umbilical circulation of the externalized human foetus reaches nearly unmeasurable levels (Heymann and Rudolph 1967, Rudolph, Heymann, Teramo, Barrett and Risha 1971). The blood gas values: the pH, pCO₂ and pO₂ in the umbilical blood vessels remain normal, but the peripheral blood gas values in the human foetus rapidly change towards acidosis: the pO₂ decreases, the pH decreases and the pCO₂ increases. Since the umbilical circulation is reduced, the distribution of the circulation in the foetus also undergoes a change: the brain, the coronary circulation and the adrenals begin to receive more blood (Rudolph *et al* 1971). The results of earlier studies on experimental animals were for the most part similar (Barker 1966, Campbell, Dawes, Fishman and Hyman 1967, Purves and James 1969).

Noradrenaline during pregnancy

NA and A disappear rapidly from the circulation as a result of tissue uptake (Pekkarinen 1948, Lund 1951, Axelrod, Weil-Malherbe and Tomchick 1959, Hertting, Axelrod and Whitby 1961, Iversen 1967). The half life of NA and A in human blood was determined to be 0.5 to 2.3 min (Cohen, Holland, Sha and Goldenberg 1959, Vendsalu 1960).

During pregnancy the disappearance of ¹⁴C-labelled di-NA and di-A does not differ from that in the non-pregnant subject. This is also true for H-labelled 1 NA (Sandler *et al* 1964, Saarikoski, unpublished data 1972). In addition, the urinary excretion of NA, A and VMA does not change during pregnancy (Castrén 1963, Zuspan, Nelson, Ahlquist and Williams 1967, Pekkarinen and Castrén 1968). Only on the day following parturition is the excretion of NA seen to be significantly elevated (Zuspan 1970, Goodall and Diddle 1971).

The female reproductive organs are adrenergically innervated. The NA concentration is highest in the ovaries and lowest in the fundal part of the uterus, where it is only about 1/4 of that in the cervix (Anton and Sayre 1962, Gaffney, Burkett and Woronkow 1965, Owman, Rosengren and Sjöberg 1967). Uterine NA concentrations increase in a number of species at the beginning of pregnancy but decrease strongly towards the end of the pregnancy (Laessle, Pekkarinen, Saarikoski and Suramo 1967, Sjöberg 1967). At present the function of the adrenergic nervous system in the puerperal organs is incompletely understood.

Methods for the determination of radioactive catecholamines and their metabolites

A number of methods have been described for the separation and physicochemical determination of CA's and their metabolites which have also been applied to the determination of ^{14}C - and ^3H labelled A, NA and their metabolites.

Extraction from tissues

Various agents have been used to extract CA's and their metabolites, for example, trichloroacetic acid, ethanol, hydrochloric acid, formic acid perchloric acid. Of these, 0.4 mol/l perchloric acid is the most widely used and it is considered best by many workers (Bertler Carlsson and Rosengren 1958, Anton and Sayre 1962, Rentzhog 1972)

Isolation and purification

Column chromatography

Aluminium oxide column: A widely used technique for CA isolation involves adsorption to aluminium oxide. Aluminium oxide binds CA's well at alkaline pH's (pH 8-8.5) but not at acidic pH's (Shaw 1938). The adsorption is specific for catecholamines and their catechol metabolites: DOPA, DA, A, NA, DHMA, DHPG, DOPAC; O-methylated metabolites: MIN, NMN, MHPG and VMA remain in the effluent. Subsequent elution is usually performed with acetic acid, formic acid or hydrochloric acid. Recovery varies from 60 to 80 %.

This procedure has also been employed for the isolation of radioactive CA's (e.g. Axelrod, Weil-Malherbe and Tomchick 1959, Sandler *et al.* 1963, 1964). The method however only provides block separation of catechol and O-methylated metabolites. If it is desired to determine NA and its principal metabolites individually additional separation techniques must be employed.

Ion exchange columns: Bergstrom and Hansson (1951) used an Amberlite IRC-50 column to separate NA and A. This resin has also been used in the determination of urinary catecholamines and their alkaline metabolites (Kirschner Goodall and Rosen 1959). The strongly acid resin, Dowex 50 is widely used for extracting CA's from tissue extracts (Bertler *et al.* 1958, Haggendahl 1962, 1963a, Iversen 1963). This resin will adsorb CA's and their O-methylated metabolites at neutral pH. Under these conditions the catechol metabolites go into the effluent. If a long Dowex column is used DA, NA, A, NMN and MIN can be eluted separately (Haggendahl 1962, Iversen 1963). The recovery with this method, with physicochemical determination, is as high as 85 to 95 %. Dowex-50 is also widely used in the determination of radioactive NA and A and of their metabolites. However it is laborious and slow. In addition, the separation of NA and all its metabolites cannot be achieved with this method alone.

Aluminum oxide columns and ion exchange resins used in succession. In 1961 Hopin, Axelrod and Gordon described a method which is now widely used for separating and determining radioactively labelled catecholamines and their individual metabolites. In this procedure, both aluminum oxide columns and Dowex 50 or Amberlite CG-50 columns are used. The free catechols are adsorbed onto aluminum oxide (Weil-Malherbe and Bone 1952) and eluted with 0.2 mol/l HCl. The conjugated catechol derivatives and O-methylated metabolites pass through with the effluent and washings. The deaminated catechols DHMA and DHPG are extracted into ethyl acetate from an aliquot of the eluent acidified with 11 mol/l HCl and saturated with NaCl. The conjugated catechols are extracted from an aliquot of the aluminum oxide effluent after acid hydrolysis.

The pH of the remainder of the aluminum oxide effluent is adjusted to 6.5 with 1 mol/l HCl and passed through a Dowex 50 ion exchange column. NMN and the other methoxyamines are eluted from the column with 3 mol/l NH₄OH. VMA and MHPG pass in the effluent. VMA is extracted from the effluent with ethyl acetate, after adjusting the pH to 1 with 6 mol/l HCl. MHPG is then extracted from the Dowex effluent, after saturation with NaCl, with ethyl acetate.

The MN, NMN and MHPG conjugates are enzymatically cleaved and determined. A similar method has also been published by Mitsuoka, Dreil, Schott, Alcaraz and James (1963). These methods have the drawback of polytomy and slow and laborious execution. Thus their application is restricted if the number of samples is large. Furthermore, the recoveries of different metabolites may vary considerably which impedes the interpretation of results.

Thin layer chromatography

Thin layer chromatography has not been used to the same extent as column chromatography in the determination of radioactive CA's. Chromatography on cellulose layers with n-butanol:3 mol/l HCl as solvent separates several CA's and CA metabolites (de Potter Vochten and de Schaepdryver 1965) as does chromatography on polyamide plates with isobutanol:acetic acid:cyclohexane (80:70:10) as solvent (Segura-Cardona and Soehring 1964). To the author's knowledge these methods have not been used for radioactive CA determination. Rentzhog (1972) has described several solvent systems for use with silica gel layers for determining acetylated CA's. Gleise R  ther and Matusek (1967) used cellulose plates with a modification of the Partridge solvent system for separating and quantitatively determining radioactive NA and its metabolites. Extraction was performed with 0.4 mol/l perchloric acid. After centrifugation, the supernatant was neutralized and applied in drops as a band across the plate. After development, the activity in the areas consistent with each metabolite was determined by scraping them into scintillation flasks. The recovery was about 96% for all metabolites. Thin layer chromatographic procedures are fast and comparatively simple. However the limited quantity of the aliquot that can be applied to each plate must be considered a drawback, as is the comparative unsuitability of the thin layer for combustion.

Paper chromatography

Paper chromatography has not been widely used in the quantitative separation of radioactively labelled CAs, although such procedures involving the use of various solvent systems have been employed for both qualitative and quantitative CA separation. The phenol-water system has been used to separate both acetylated and unacetylated CA metabolites and amines (James 1948, Crawford 1951, Goldstein, Friedhoff and Simmons 1959). The most widely used system for separating catecholamines and their metabolites (James and Milby 1950) is Partridge's solvent (butanol:acetic acid:water 4:1.5 or 4:1:1 or 4:1:2) (Partridge 1948). Schanberg *et al.* (1968b) used two different solvent systems butanol:ethanol:water and Partridge's solvent, in their studies of the metabolism of ^3H -NMN in rat brain. After p-nitroaniline staining, strips 1 cm in width were placed in a phosphor-ethanol (9:1) solution and the tritium was determined by liquid scintillation counting, thus providing a quantitative paper chromatographic method. Many workers use paper chromatography qualitatively in association with quantitative CA analysis based on column chromatography etc. as an additional parameter for identifying the radioactive peak with known metabolites (e.g. Hopin *et al.* 1961, LaBrosse *et al.* 1961). Paper chromatography has also been used in the examination of the purity of ^3H -labelled catecholamines.

Rutschmann, Pacha, Halberer and Schreier (1965) have described a method based on two-dimensional paper chromatography for studying catecholamine metabolism. They used Whatman no. 1 paper and n-butanol saturated with 1 mol/l HCl (v. Euler and Hamburger 1949) and nitroethane:70% acetic acid (9:4) as solvents. With this method they were able to determine ten different catecholamines and catecholamine metabolites: DOPA, DA, NA, A, MT, NMN, MN, DHMA, VMA and HVA.

CAs and their metabolites can be efficiently separated using paper chromatography, but elution of these substances from the paper for further physicochemical or radiochemical analysis has many attendant difficulties. However, when paper chromatography is coupled with the incineration method the problems of elution are overcome, and a good counting efficiency is obtained.

PROBLEMS AND PURPOSE OF THE STUDY

The adrenergic nervous system plays a central role in the regulation of the foetal circulation under normal conditions and in crisis situations, e.g. in asphyxia. The information available on NA biochemistry in relation to the foetoplacental unit is sparse and that concerning the dynamic aspects of NA metabolism is totally lacking. It has not either been clarified with any certainty whether the NA in the mother's blood affects the foetal circulation or whether the effects induced in the foetus by catecholamine administration to the mother in fairly high doses are merely reflections of actions on the uterine blood vessels and the uterine muscles. In the assessment of the functional status of the adrenergic nervous system in the human foetus in various physiological and pathological situations the main difficulty arises from the scarcity of fundamental data. Additional studies would thus seem essential.

The object of the present study was.

- 1 To develop a simple reliable and fast method for the analysis of radioactively labelled NA and its metabolites under conditions where it is desirable to use small amounts of radioactivity for instance in human experimentation
- 2 To study the rate of passage of NA and its metabolites through the placenta.
- 3 To study the ability of foetal tissues to take up store and metabolize NA and its metabolites.
- 4 To study the ability of the foetus to conjugate NA and its metabolites.

MATERIAL AND METHODS

Foetuses

The foetuses used in this study were obtained during the legal interruption of pregnancy. None of the patients suffered from any acute physical disease. The majority of the pregnancy interruptions were made for social, and partly with psychiatric reasons. Foetal age was determined from the mother's last menstruation, the size of the uterus, the foetal weight and the CR length (see Hervonen 1971). The material is described in Tables 1 and 2. The duration of pregnancy in the series varied from 11 to 24 weeks and the weights of the foetuses, from 15 to 420 g.

The purpose of the study and the procedures involved were explained to every woman prior to surgery and each subject consented to participate. The procedures associated with the study did not prolong the hospitalization period and did not impede recovery.

The study comprises four parts, identified in the results section with the Roman numerals I to IV.

Anaesthesia

As premedication, prior to surgery the women were given atropine 0.1 mg per 10 kg i.m. and diazepam 10–15 mg i.m. (in partial studies I and II) or instead of the latter 25 mg promethazine chloride and 50 mg pethidine i.m. (in parts III and IV). Anaesthesia was induced with thionembutal, which was administered intravenously. The anaesthesia was continued with halothane at a concentration of 0.5% and 2 l mixture of nitrous oxide and oxygen in order to avoid contraction of the uterus prior to sampling. For intubation, the patients were given succinylcholine chloride as a muscular relaxant, followed by d-tubocurarine or alkuron chloride.

Delivery of the foetus

All discontinuations of pregnancy were performed by laparotomy. The abdominal covers were entered through a Pfannenstiel or low transverse incision. The muscular layer and peritoneum were opened longitudinally. The uterus was opened by trans-

TABLE 1 *Age and weight of foetuses.*

No.	Weight, g	CR cm	Week of gestation	Partial Study
1	15		11	III
2	16	5.9	12	II
3	16	7.2	13	II
4	20		12	III
5	22	6.0	12	II
6	33		13	III
7	35	8.5	14	IV
8	42	8.7	14	II
9	42	9.4	14	II
10	47	9.5	14	II
11	48	9.2	14	II
12	58	10.4	15	II
13	60	10.4	15	II
14	60	10.6	15	II
15	60		15	III
16	62		15	III
17	62	9.5	15	II
18	63	9.8	15	II
19	66	10.2	15	IV
20	70	11.4	16	II
21	70		16	III
22	73		16	III
23	74	10.8	16	IV
24	75		16	III
25	76	11.6	16	I
26	85	11.4	16	I
27	86	11.0	16	II
28	87		16	III
29	88	11.6	16	I
30	94	11.7	16	III
31	110	12.5	17	II
32	115	11.9	17	II
33	140	14.1	18	I
34	147	14.4	18	II
35	157	14.6	18	IV
36	160	15.1	19	I
37	206	16.9	20	II
38	221		20	III
39	253	16.5	20	III
40	253	16.0	20	IV
41	260	16.2	20	IV
42	274		20	III
43	350	18.6	22	I
44	470	22.0	24	I

TABLE 2 Age and weight of mothers. Mothers given ^3H -NA into their antecubital vein included.

Age, years	n	Weight, kg	n
<20	5	<50	2
20-30	9	50-60	13
30-40	8	60-70	8
>40	3	>70	2

verse incision in the lathmic part. A sample of the amniotic fluid was taken prior to entering the uterus, and a specimen of uterine muscle from the margin of the uterine incision.

The foetus was always removed from the uterus. Attempts were made to keep the umbilical cord free, and its manipulation was confined to a minimum. When working on the foetoplacental unit the foetus was covered with a cloth saturated with saline for the duration of the experiment.

Administration of radioactive substances

l-Noradrenaline-7 H of high specific activity was used in the study except in part III where also dl-noradrenaline 7- ^3H was employed. The material was in aqueous solution as the acetate and had a specific activity between 5.8 and 8.7 Ci/mmol (obtained from the Amersham Radiochemical Centre, England). The ^3H labelled NA diluted with physiological saline was injected either into the mother's antecubital vein (parts I and II) into the umbilical vein of the foetus (parts III and IV) or into the jugular vein of the foetus (part III). The administration of the radioactive substrate and the sampling procedure are described in each part of the study.

In this study the plasma NA concentration at the time of ^3H NA injection was not determined, nor was the endogenous NA concentration in the maternal or foetal blood or in the foetal tissues determined in connection with the individual samples. The peripheral NA concentration is believed to reflect the NA overflow from the adrenergically innervated tissues. Changes in plasma NA concentrations are thought to reflect changes in the activity of the adrenergic nervous system in the tissues (see Axelrod 1965, Iversen 1967).

It is known from previous studies that under standardized at rest conditions the changes in plasma NA concentration are comparatively minimal, and fluorothane or thionembutal/nitrous oxide/oxygen anaesthesia does not significantly change the plasma NA concentration even during the operation (Hamelberg, Sprouse, Mahaffey and Richardson 1960, Nikkí Takki Tammisto and Jaattela 1972). In the last of the studies cited, the mean plasma NA concentration of 11 abdominal surgical patients was 0.66 $\mu\text{g/l}$ and the coefficient of variation was 30.0%. The variation in the values obtained for replicate samples with the method used was $\leq 10\%$ (Tammisto, Jaattela, Nikkí and Takki 1971). The samples taken from five patients under at rest con-

ditions during pregnancy showed a mean plasma NA concentration of 0.65 $\mu\text{g/l}$ with a coefficient of variation of 33.3 % (Saarikoski, Jäätelä and Ikonen unpublished data 1972)

In the present study the H NA and metabolite concentrations in the foetus and its mother are compared. This reduces the influence of dosage variation on the results. The quantities of blood obtained from the foetuses were so small, from 0.5 to 1.5 ml, that they would not suffice for the determination of the endogenous content of NA and its metabolites by conventional spectrofluorometric methods.

The reliable counting efficiency for H activity is very low. According to the International Commission on Radiological Protection the maximum safe daily dose of H is 1000 μCi . The dosage used here was 50 or 100 μCi , between 1/10 and 1/20 of the latter.

The radiation effect produced by the administered radioactivity also depends on the biological half-life of the substance in the organism. The radiation dose can be determined from the formula $D_{\beta}(\infty) = 73.8 \cdot C \cdot \bar{E}_{\beta} \cdot T_{1/2}$ rad (Johns 1971) where C stands for the activity per unit mass, \bar{E}_{β} for the mean energy of the beta particles, and $T_{1/2}$ for the biological half-life of the substance. It was found in the present study that of the H activity administered $43.1 \pm 4.1\%$ was excreted in the urine during the first 24 hours. If the biological half-life is taken to be 27 hrs (see Iversen 1967) the patient's weight 60 kg and the dose 100 μCi , the radiation dose with reference to the whole body is $D(\infty) = 73.8 \cdot (100/60,000) \cdot 0.006 \cdot (27/24) = 0.83$ mrad. Such a dose is very small and does not endanger the patient in any way.

Chemicals

For treatment of tissues. Perchloric acid (E. Merck AG) l-ascorbic acid (E. Merck AG) ethyl alcohol A7 (Oy Alko Ab) Glusulac[®] (Endo Laboratories) Ketodac[®] (Warner Lambert Pharmaceutical Company)

For paper chromatography. n-butanol (E. Merck AG) formic acid (E. Merck AG) butyl formate (Fluka AG) glass distilled water p-nitroaniline (E. Merck AG) H_2CO (E. Merck AG) NaNO (E. Merck AG)

Cl and metabolites. l-adrenaline hydrochloride (Fluka AG) l-noradrenaline-d-hydrogen tartrate (Fluka AG) dl-metanephrine hydrochloride (Sigma Chemical Company) 3,4-dihydroxyphenylglycol (Sigma Chemical Company) dl-3,4-dihydroxymandelic acid (Sigma Chemical Company) 3-methoxy-4-hydroxyphenylglycol, piperazine salt (Sigma Chemical Company) dl-3-methoxy-4-hydroxymandelic acid or anilhyldandelic acid (Sigma Chemical Company) 3-methoxytyramine hydrochloride (Sigma Chemical Company) 3,4-dihydroxyphenylalanine (Fluka AG) dopamine (3-hydroxytyramine hydrochloride Fluka AG) anillic acid (3-methoxy-4-hydroxybenzoic acid Sigma Chemical Company) homovanillic acid (Calbiochem) 3,4-dihydroxyphenylacetic acid dicyclohexylamine salt (Calbiochem)

For liquid scintillation counting. dioxane (E. Merck AG) toluene (E. Merck AG) naphthalene (E. Merck AG) 2,5-diphenyloxazol (PPO New England Nuclear Chemicals GmbH) P bis[2 (4-methyl-5-phenyloxazolyl)]-benzene (dimethyl-POPOP New England Nuclear Chemicals GmbH) dl-nor-metanephrine-7 H (in 0.1 mol/l acetic acid, specific activity 3.2 Ci/mmol, New England Nuclear Chemicals GmbH)

Extraction

Blood and tissue NA and NA metabolites were extracted with 0.4 mol/l perchloric acid. To facilitate staining 1 mg NA, 0.5 mg NMN, 1.0 mg DHMA and DHPG and 0.5 mg MHPG and VMA were added to the perchloric acid in an ice bath. To the samples from the adrenals and aortic bodies 1.0 mg A and 0.5 mg MN also were added. The perchloric acid contained ascorbic acid (1 mg/10 ml) as an antioxidant.

After placing the tissues and blood in pre weighed tubes containing perchloric acid, they were rapidly cooled in an ice bath by adding ethanol to the crushed ice. This lowers the temperature to between -15 and -20°C . After weighing the tissues were homogenized in an Ultra Turrax (Tp 18/2 Janke & Kunkel KG) homogenizer.

Not sooner than 30 min after homogenization the samples were centrifuged for 20 min at $10\,000 \times g$ at $+4^{\circ}\text{C}$ and the supernatant collected for the determination of NA metabolites and total activity.

The samples were neutralized with 2 mol/l K_2CO_3 (to pH 5.4–5.8). Usually about 70 μl of K_2CO_3 per ml of supernatant was required.

Paper chromatography procedure

The paper chromatographic method employed was developed by the author and has not been published in detail, although it was used in some earlier studies (Castrén *et al* 1968, Castrén and Saarikoski 1969, 1974).

Whatman no. 1 paper was used. The solvent system used was n butanol:formic acid:butyl formate:water (7:1:2 v/v) freshly prepared, or a two-day-old mixture n butanol:formic acid:water 70:12:15 v/v.

An aliquot of the supernatant was spotted dropwise across the lower margin of a paper strip 9×30 cm and raised as a thin band to a height of about 4 cm from the lower margin with distilled water using the so-called running up technique. The volume of the aliquot spotted was between 0.5 and 2.5 ml, most often 1.5 ml. The paper strips were then suspended in the chromatography tank over the running solvent to equilibrate for 18 to 36 hrs, usually 24 hrs. The chromatograms were developed for 12 hrs in an ascending fashion at room temperature. If it was desired to determine NA/A and NMN/MN separately descending chromatography for 18 to 22 hrs was employed.

TABLE 3. *Rf* values of NA and major metabolites. Paper: Whatman no. 1; solvent: n-butanol:formic acid:water 0:12:15 v/v 2 days old, temperature 22°C, equilibration time: 24 hrs, ascending time: 12 hrs.

Compound	<i>Rf</i> value
NA	0.18
NAN	0.34
DHPG	0.49
DHMA	0.56
MHPG	0.68
VMA	0.75

TABLE 4. *Rf* values of catecholamines and their metabolites. See legend to Table 3.

Compound	<i>Rf</i> value
NA	0.18
DOPA	0.19
A	0.23
NAN	0.34
MAN	0.38
MT	0.46
DHPG	0.49
DHMA	0.56
MHPG	0.68
VMA	0.75
DOPAC	0.80
HVA	0.90
VA	0.93

The chromatographic strips were dried after the run. They were then stained by spraying with a p-nitroaniline reagent. 1 part 0.1% p-nitroaniline (100 mg p-nitroaniline solved in 2 ml of conc. hydrochloric acid and diluted with 98 ml of distilled water) and 1 part 0.2% NaNO₂ were mixed and kept at +4°C for 5 min and finally 2.5 parts 10% H₂CO₃ was added immediately before use. The *Rf* values of the CA and CA metabolites in this system are given in Tables 3 and 4 and the chromatograms are shown in Figs 1 and 2.

NA and the catechol metabolites DHMA and DHPG stain light blue and the methoxy derivatives NAN, MHPG and VMA violet. The metabolites are easily differentiable.

Determination of total activity: noradrenaline and its metabolites

The total ³H content in the tissues was determined by applying part of the neutralized extract in drops to the lower margin of a Whatman no. 1 paper. After drying the paper strip was combusted using the sample oxidizer of Kaartinen (1969) and the quantity of ³H₂O produced was determined by liquid scintillation counting. The scintillation

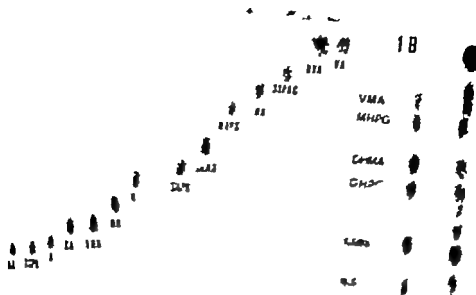


Fig. 1. Chromatogram of esters and isomers shows the behaviour of 14 compounds on 100-100 mesh from separate start point. — Fig. 1B, A includes all applied at the same point. Fig. 1B applied at the same starting point. Paper V7, n-butylformic acid: water 0:12.15 2/4 22 C, equilibration time: 12 hrs. Stained with



Fig. 2. Chromatogram of nitrobenzidine and major metabolites from neutralized placental homogenate was applied on Whatman 3001 paper and developed with water to height of about 4 cm. The solvent: n-butanol: formic acid: butyl formate = 10:10:1:1. Equilibration time 24 hrs, ascending time 12 hrs. Nitrobenzidine reagent, NA, DHPG and DEMA are stained blue and MA, MHPG and MA are stained red.

samples free of activity in alternation. The average retention effect from nine samples and the corresponding blanks was $0.18 \pm 0.08\%$ (mean \pm S D). The results are consistent with those reported by Kaartinen (1969).

Chromatography

Recovery of total activity The total activity recovery was determined by adding a known quantity of ^3H NA or ^3H NMN to a tissue homogenate. After centrifugation, the supernatant was neutralized and applied to the lower margin of the paper and dried. The sample was then burned. The activity recovered was compared with that obtained by adding an equivalent amount of radioactivity directly to the scintillation liquid. The mean of 14 determinations was $100.7 \pm 2.1\%$ (mean \pm S D). It can thus be seen that no activity is lost in the centrifuging and neutralizing operations.

Recovery of metabolites The recovery of ^3H NA and its metabolites from the paper chromatography procedure without hydrolysis and after hydrolysis was determined by adding known quantities of ^3H NA, ^3H NMN, ^3H DHPG, DHMA or ^3H MHPG-VMA to foetal tissue homogenates of heart, liver, placenta, lung, brain, blood or kidney. In some of the tests a mixture of several kinds of tissue was used. Since no ^3H labelled DHPG, DHMA, MHPG and VMA were available, these were prepared by incubating 1 ^3H NA or di- ^3H NMN with placental homogenates for 15 min as previously described (Castrén and Saarikoski 1969, 1974). The metabolites were isolated and purified using the paper chromatographic process employed in this study and were eluted with 0.1 mol/l acetic acid. The eluates of interest contained ^3H DHPG and ^3H DHMA in proportions 3:1 and ^3H MHPG and ^3H VMA in proportions 2:1 respectively. For chromatography without hydrolysis, the recovery values for NA and its metabolites (94.1–97.4, Table 5) did not differ at any statistically significant level. This was also the case for the recovery values obtained when the same hydrolytic procedure was included. The percentage of the activity spreading out on the paper *ad flandum* is rather equally distributed between the different metabolites. No corrections for the procedural losses incurred were made. The recovery of conjugates was not determined.

TABLE 5. *Per cent recovery after paper chromatography of ^3H -NA and its metabolites.* The recovery of ^3H -NA and of its metabolites was determined by adding a known quantity of ^3H -NA or metabolite to a foetal tissue homogenate. The recovery was determined after chromatography without hydrolysis (I $n = 9-10$) after acid hydrolysis for 15 min at 100°C in 0.4 mol/l perchloric acid (II $n = 6-10$) after sulphatase + β -glucuronidase hydrolysis for 24 hrs at 37°C (III $n = 0-9$) and after β -glucuronidase hydrolysis for 24 hrs at 37°C (IV $n = 6-9$)

Compound	I	II	III	IV
NA	94.1 ± 3.4	85.9 ± 3.5	86.0 ± 5.0	85.9 ± 4.1
NMN	97.4 ± 2.0	88.6 ± 3.0	94.5 ± 1.9	95.2 ± 0.7
DHPG-DHMA	96.6 ± 3.0	89.4 ± 1.7	94.4 ± 1.7	93.5 ± 0.5
3HHPG-VMA	94.4 ± 3.5	85.9 ± 4.4	91.6 ± 1.1	90.2 ± 2.2

Reproducibility of the paper chromatography procedure When seven replicate runs were made with the same foetal liver tissue homogenate, the percentage of the total activity found for the individual metabolite fractions was constant: the S.D. of the different metabolite values was between 0.5 and 2.0 % of the total activity. The mean values (in dpm) and coefficients of variation for the NA and metabolite fractions were: start, $57 \text{ dpm} \pm 20.4\%$ conjugates, $155 \text{ dpm} \pm 4.9\%$ NA, $58 \text{ dpm} \pm 12.1\%$, NMN $34 \text{ dpm} \pm 23.8\%$ DHPG-DHMA $45 \text{ dpm} \pm 23.3\%$ 3HHPG-VMA, $420 \text{ dpm} \pm 6.1\%$ and front $22 \text{ dpm} \pm 16.4\%$. The coefficient of variation was highest for those metabolites which contained the lowest activity.

Loss of activity during chromatography The aggregate activity (in dpm) of all the metabolites in a liver sample determined in seven replicate runs did not differ from the total values obtained without chromatography. After chromatography $99.2 \pm 4.2\%$ (mean \pm S.D.) of the amounts determined without chromatography were obtained.

Comment

In the paper chromatography procedure described above the separation of NA and its metabolites is very well defined. The conjugates have R_f values between 0.00 and 0.03 and are thus completely separated from NA. Only DHPG and DHMA on the one hand and 3HHPG and VMA on the other run close together. However their separation is often of little significance in studies of NA kinetics. The method presented based on paper chromatography is rapid and simple. In addi-

tion it is suitable for the analysis of large numbers of samples. The large aliquot volume that can be applied to the paper is also a great advantage in that it allows lower activities than before to be measured reliably. The recoveries of different metabolites are reasonably equal. A similar paper chromatography method (Rutschmann *et al.* 1965) had a greater number of steps and included lyophilizing steps which are very time-consuming. The method employed by Kopin *et al.* (1961) is rather more complicated, and the recoveries of individual metabolites vary considerably. The recovery values obtained with the present method compete well with those obtained with the cellulose plate chromatography method of Giese *et al.* (1967).

RESULTS

1 Rate of passage of ^3H noradrenaline and its metabolites through the placenta

Material and methods

$1\text{-}^3\text{H}$ -NA, $2.8\text{ }\mu\text{g}$ or $100\text{ }\mu\text{Ci}$, was administered to seven mothers over a period of 10 seconds into the antecubital vein under general anaesthesia at the stage when the abdominal covers had been opened and the uterus was visible. The uterus was cut open 45 seconds after injection. Blood samples were taken simultaneously from the mother's peripheral blood, from the arm contralateral with the point of injection and from the umbilical vein and umbilical artery of the foetus 1 min, 2.5 min, 3.5 min and 5 min after injection. From one mother no blood samples were obtained and from one foetus only one sample. The blood that was drawn was pipetted into 0.4 mol/l perchloric acid at 0°C and cooled. The metabolites and total activity were determined as described in the section on methods.

Results

The results are shown in Tables 6–9 and in Figs 3–4. The ^3H activity rapidly disappeared from the maternal blood and a measurable amount of ^3H activity was already present in the umbilical blood of the foetus 1 min after the injection. About 3.5 min after the injection an equilibrium appeared to exist between the radioactivity in the umbilical venous blood and the mother's peripheral blood. The ^3H activity in the umbilical arterial blood 1–5 min after the injection was so low that it could not be ascertained with any certainty.

The percentage of the total activity in the maternal blood present as ^3H NA decreased rapidly: 5 min after injection only about 25% of the ^3H NA remained unmetabolized in the blood. In the umbilical venous blood of the foetus the ^3H NA level was very low: it was mark

TABLE 6 *HNA and its metabolites in maternal peripheral blood and fetal umbilical venous blood 1 min after HNA administration to the mother* During anaesthesia 2.8 µg or 100 µCi, of 1 H-NA were injected over 10 sec 1 to the mother's antecubital vein. Samples were taken from the arm contralateral from the injection, and simultaneously from the umbilical vein of the foetus. NA and metabolite quantities stated in Ci/g, metabolites in the umbilical blood also as percentage of the corresponding maternal metabolite (mean ± S.E.M) Number of determinations in parentheses.

Compound	Maternal blood (5)	Umbilical venous blood (6) (4)	
	nCi/g	nCi/g	of corresponding maternal value
Start	0.64 ± 0.22	0.06 ± 0.03	10.6 ± 2.2
Conjugates	0.74 ± 0.11	0.02 ± 0.00 ^a	3.5 ± 0.8
NA	4.22 ± 1.32	0.04 ± 0.00 ^a	1.2 ± 0.2
NALN	0.27 ± 0.09	0.01 ± 0.00	5.6 ± 2.6
DHPG-DHMA	0.11 ± 0.01	0.04 ± 0.01	54.8 ± 25.6
MHPG-VMA	0.09 ± 0.01	0.12 ± 0.07	170.9 ± 106.3
Front	0.08 ± 0.03	0.05 ± 0.03	55.5 ± 44.0
p	0.05	difference versus maternal value	
p	0.01		
p	0.005		

TABLE 7 *11-NA and its metabolites in maternal peripheral blood and fetal umbilical venous blood 2.5 min after 11-NA administration to the mother* See legend to Table 6.

Compound	Maternal blood (5)		Umbilical venous blood (6) (5)	
	nCi/g		of corresponding maternal value	
Start	0.36	0.06	0.14 ± 0.08	50.7 ± 20.7
Conjugates	0.37	± 0.05	0.08 ± 0.03 ^a	30.5 ± 10.7
NA	1.57	0.32	0.10 ± 0.02 ^a	7.6 ± 2.1
NALN	0.16	0.02	0.06 ± 0.02	56.0 ± 17.1
DHPG-DHMA	0.12	0.03	0.23 ± 0.07	447.8 ± 269.0
MHPG-VMA	0.13	0.02	0.83 ± 0.30 ^a	861.6 ± 374.1
Front	0.05	0.02	0.06 ± 0.04	161.9 ± 67.9
p	0.01-0.05 difference versus maternal value			
p	0.01			

TABLE 8. *HVA and its metabolites in maternal peripheral blood and fetal umbilical venous blood 3.5 min after HVA administration to the mother. See legend to Table 6.*

Compound	Maternal blood (6)	Umbilical venous blood	
	nCi/g	(6)	(3) % of corresponding maternal value
Start	0.21 ± 0.04	0.15 ± 0.08	71.9 ± 24.8
Conjugates	0.27 ± 0.05	0.15 ± 0.02 ^a	50.9 ± 8.5
VA	0.83 ± 0.17	0.12 ± 0.02 ^a	19.1 ± 8.2
VAN	0.18 ± 0.02	0.09 ± 0.02	50.3 ± 9.4
DHPG-DHMA	0.11 ± 0.01	0.21 ± 0.05	291.7 ± 109.9
MHPG-VMA	0.18 ± 0.02	0.97 ± 0.99 ^a	601.5 ± 155.4
Free	0.04 ± 0.01	0.07 ± 0.04	122.9 ± 51.3

^a $p = 0.1-0.05$ difference versus maternal value

$p < 0.05$

$p < 0.01$

TABLE 9. *HVA and its metabolites in maternal peripheral blood and fetal umbilical venous blood 5 min after HVA administration to the mother. See legend to Table 6.*

Compound	Maternal blood (6)	Umbilical venous blood	
	nCi/g	(3)	(4) % of corresponding maternal value
Start	0.24 ± 0.06	0.09 ± 0.02	37.6 ± 14.4
Conjugates	0.30 ± 0.05	0.09 ± 0.02 ^a	32.4 ± 10.9
VA	0.41 ± 0.07	0.09 ± 0.03 ^a	23.6 ± 9.8
VAN	0.18 ± 0.03	0.08 ± 0.04	52.0 ± 22.0
DHPG-DHMA	0.14 ± 0.02	0.14 ± 0.05	103.7 ± 68.4
MHPG-VMA	0.20 ± 0.04	0.66 ± 0.28	637.5 ± 261.6
Free	0.06 ± 0.02	0.04 ± 0.01	194.7 ± 64.3

^a $p < 0.05$ difference versus maternal value

$p < 0.01$

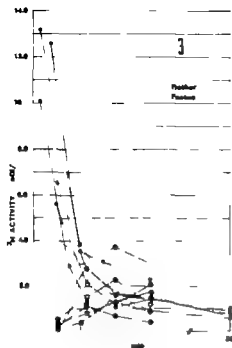


Fig. 3. Total H concentration in maternal blood and fetal umbilical venous blood after administration of HNA to the mother. During anaesthesia, 2.8 μ g, or 100 μ Ci of $1\text{-}^3\text{H}$ -NA was injected over 10 sec into the mother antecubital vein. Samples were taken from the mother's peripheral blood, and simultaneously from the umbilical vein of the foetus. Individual values are shown in the figure. From one mother no samples were obtained, and from one foetus only one sample. The results are given in nCi/g.

edly lower than that in the mother's peripheral blood ($p < 0.01$) and represented only about 10 % of the total at any time. The amount present remained unchanged from 1 min onwards. However the proportion of the total activity in the umbilical venous blood present as ^3H NA, relative to the amount in the mother's peripheral blood increased from 1 % to about 25 % as the ^3H NA concentration in the maternal blood decreased.

The per cent distribution of ^3H NA metabolites in the foetal blood differed greatly from that observed in the maternal blood. In the mother's blood the relative amounts of ^3H NMN, ^3H DHPG, DHMA and ^3H MHPG-VMA increased uniformly with time, while in the foetal blood the level of ^3H NMN remained constant at 5 % of the total activity and did not reach the level detected in the maternal blood. The percentage from the total activity present as ^3H DHPG, DHMA and ^3H MHPG-VMA, combined was already 45 % 60 seconds after the injection and 65–75 % subsequently. The level of ^3H MHPG-VMA was higher, on a percentage basis, than in the maternal blood at all times. The amount of activity present in conjugated form was higher in the maternal than the foetal blood ($p < 0.05$ or $p < 0.005$).

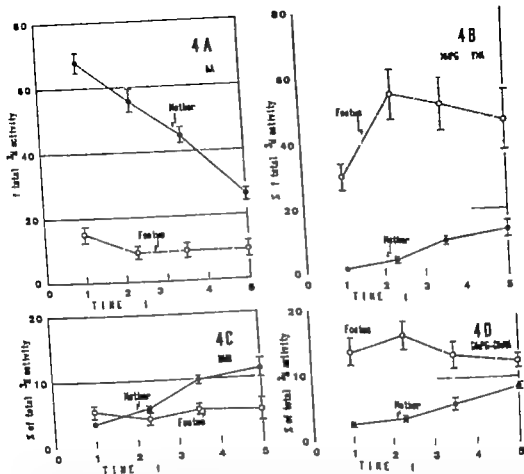


Fig. 4 Presence of HNA and its metabolites in maternal blood and fetal umbilical venous blood 1-5 min after HNA administration to the mother. Quantity of metabolites expressed as percentage of total activity (mean \pm S.E.M.) — Fig. 4A: Quantity of NA, Fig. 4B: Quantity of MHPG-VMA, Fig. 4C: Quantity of NMN, Fig. 4D: Quantity of DHPG-DHMA. For other particulars, see legend to Fig. 3.

Comment

In the present study only some 10 % of the ^3H activity was present in the umbilical vein of the foetus as HNA. Thus, little noradrenaline may pass, intact, through the placenta, like it was found by Sandler *et al* (1963 1964)

The results show that HNA is very rapidly metabolized as it traverses the placenta. For ^3H MHPG-VMA production the ^3H NA metabolism must be mediated both by COMT and MAO. Since the

centrations of ^3H MHPG VMA were higher in the foetus than in the mother the metabolism obviously takes place in the placenta. The results were not very much affected by ^3H NA metabolism in the foetus, as the activities in the umbilical artery were very low.

Manipulation of the umbilical cord rapidly weakens the umbilical circulation as does the extraction of the foetus from the uterus (Rudolph *et al.* 1971). It was obvious that foetal asphyxia developed during the procedure, but its strength was not measured. Frequently however blood sampling after 5 min was already difficult, owing to closure of the umbilical blood vessels.

II Distribution of ^3H noradrenaline and its metabolites in foetal tissues after i.v. administration of ^3H noradrenaline to the mother

Material and methods

1- ^3H NA, 1.2 μg or 50 μCi , was injected into the mother antecubital vein during anaesthesia. The samples were taken 5, 30 and 240 min after injection. In the case of those patients from whose foetus samples were taken after 5 min the injection was made at the stage when the abdominal covers including the peritoneum had been opened and the uterus exposed.

Those mothers whose foetus was sampled after 30 min received the ^3H NA injection before the commencement of the operation and those with a sampling time of 240 min, prior to premedication and operation.

After opening the uterus, the foetus was removed and blood samples were taken from the umbilical vein and artery and simultaneously from the mother's arm on the side contralateral with the point of injection. The foetal tissues were dissected in the following sequence: the heart was first removed, then the lungs, liver, intestine, orotic bodies, adrenals, kidneys, brain and brain stem and if possible, foetal urine was collected. In a few instances a sample of the amniotic fluid was taken before entering the uterus. The placental sample was taken last of all.

Both the 5-min and 30-min groups consisted of six subjects, and the 240-min group of four. The duration of pregnancy assessed by the size of the foetus, was from 11 to 40 weeks.

Results

Total activity

Blood. The ^3H concentrations in the maternal blood and in the umbilical vein and artery of the foetus are shown in Fig. 5 where the

means have been plotted and the vertical lines indicate the S.E.M. The ^3H activity disappears very rapidly from the maternal blood. The 1 min values were derived from mothers whose foetus was extracted 30 min after the injection. The individual values in two 1 min foetal blood samples are shown. Five, 30 and 240 min after injection the ^3H activity in the maternal blood and the umbilical vein blood was in equilibrium, and the levels were not significantly different.

The ^3H activity in the umbilical artery was significantly lower ($p < 0.005$) than that in the umbilical vein 5 min after injection, but after 30 and 240 min an equilibrium prevails between the ^3H concentrations in the umbilical vein and artery.

The rate of removal of the ^3H activity from the circulation during anaesthesia was not significantly different from that in the patients who received the injection prior to anaesthesia (Fig. 6)

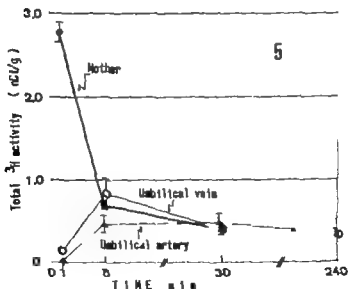


Fig. 5. Total ^3H concentration in maternal blood and foetal umbilical venous and arterial blood after ^3H -NA administration to the mother. During anaesthesia, 1.2 μg or 50 μCi of ^3H -NA were injected over 10 sec into the mother's antecubital vein when samples were taken 5 or 30 min after the injection, and similarly prior to anaesthesia for sampling after 240 min. — ^3H activity is given in nCi/g. Only 2 foetal samples were obtained 1 min after the injection, other samples numbered between 4 and 10 per time interval, and the S.E.M. is given in these cases.

Foetal tissues and placenta. The placenta contained a greater proportion of the ^3H activity than the foetal tissues (Tables 10—12). The placental activity was between 10 and 40 times the ^3H content of the liver. The distribution of the total activity 5 and 30 min after the injection was for the most part similar: the liver had received the greatest share, followed by the lungs, hemispheres, intestine and kidneys. The total activity in the aortic bodies was lowest. Four hours after injection the ^3H content of the lungs was highest, and the activity in the hemispheres had risen to about 80 % of that in the liver.

The activity (in nCi/g) in the tissue is shown in Tables 10—12. The activity of the liver 5 min after the injection, was 0.88 ± 0.15 which is significantly higher than that in the placenta, lungs, heart and brain and brain stem ($p < 0.01$).

The activity in the liver 30 min after the injection, 2.10 ± 0.31 is significantly ($p < 0.01$) higher than at 5 and 240 min. The ^3H activity in the liver was higher than that in the heart, lungs, intestine, brain and brain stem ($p < 0.05-0.01$). The activity in the brain and

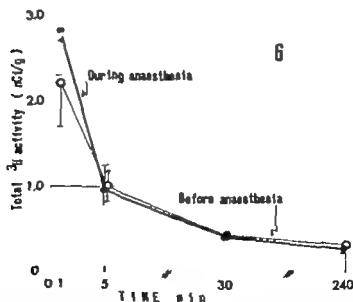


Fig. 6. Effect of anaesthesia on disappearance of ^3H from the blood stream. The figure is a comparison between the rate of disappearance of ^3H NA injected prior to anaesthesia and during anaesthesia. — Radioactivity in nCi/g mean \pm S.E.M. of 4—10 determinations. For other particulars, see legend to Fig. 5.

brain stem was lowest on the average at all times, and there are no statistically significant differences in the 5, 30 and 240-min values. The ^3H activity in the placenta was also constant throughout the observation period. After 240 min, the ^3H activity in the aortic bodies was highest, 4.98 ± 0.33 while the activities in the other tissues were almost equal and close to their respective 5 min values. Comparison of the

TABLE 10. Concentration and content of ^3H in placenta and fetal tissues 5 min after ^3H NA administration to the mother. Mean \pm S.E.M. of 5-6 determinations.

Time	H concentration nCi/g	% of ^3H concentration in the liver	Total H content/tissue, nCi	% of ^3H content in the liver
liver	0.88 ± 0.15	100.0 ± 0.0	2.88 ± 0.85	100.0 ± 0.0
placenta	0.62 ± 0.16	69.9 ± 8.9	56.95 ± 13.28	2254.6 ± 286.8
heart	0.41 ± 0.08	50.5 ± 11.3	0.23 ± 0.13	6.4 ± 1.5
lungs	0.24 ± 0.06	27.6 ± 8.9	1.26 ± 0.82	30.6 ± 10.1
intestine	0.53 ± 0.30	50.7 ± 17.5	1.23 ± 0.56	34.0 ± 8.9
aortic bodies	1.24 ± 0.84	134.9 ± 130.6	0.06 ± 0.05	1.2 ± 0.7
adrenals	0.52 ± 0.14	90.3 ± 47.8	0.27 ± 0.16	10.6 ± 5.6
kidneys	0.82 ± 0.07	116.1 ± 29.1	0.67 ± 0.25	26.8 ± 9.0
brain stem	0.08 ± 0.00	7.6 ± 1.8	0.11 ± 0.03	5.2 ± 1.3
hemispheres	0.12 ± 0.03	14.6 ± 5.8	1.87 ± 1.43	39.6 ± 18.6

TABLE 11. Concentration and content of ^3H in placenta and fetal tissues 30 min after ^3H NA administration to the mother. Mean \pm S.E.M. of 5-6 determinations.

Time	^3H concentration, nCi/g	% of H concentration in the liver	Total ^3H content/tissue, nCi	% of H content in the liver
liver	2.10 ± 0.31	100.0 ± 0.0	4.51 ± 0.97	100.0 ± 0.0
placenta	0.59 ± 0.07	30.4 ± 5.4	42.46 ± 5.43	1079.9 ± 146.6
heart	0.91 ± 0.14	44.8 ± 8.1	0.29 ± 0.09	6.5 ± 1.7
lungs	0.95 ± 0.08	34.5 ± 7.8	2.13 ± 0.86	40.2 ± 9.7
intestine	0.68 ± 0.14	34.1 ± 7.2	0.90 ± 0.30	20.1 ± 5.9
aortic bodies	5.80 ± 1.30	224.0 ± 86.4	0.16 ± 0.04	3.6 ± 1.0
adrenals	1.27 ± 0.22	63.9 ± 11.8	0.33 ± 0.11	6.9 ± 1.1
kidneys	1.42 ± 0.18	73.3 ± 11.2	0.69 ± 0.20	14.0 ± 2.3
brain stem	0.31 ± 0.04	16.1 ± 3.7	0.45 ± 0.09	11.7 ± 2.6
hemispheres	0.29 ± 0.06	13.7 ± 1.7	1.63 ± 0.40	33.5 ± 5.3

TABLE 12 Concentration and content of H in placenta and foetal tissues 10 min after HNA administration to the mother Mean \pm S.E.M. of 4 determinations.

Tissue	H concentration, nCi/g	% of H concentration in the liver	Total H content/tissue, nCi	% of 3H content in the liver
liver	0.50 ± 0.10	100.0 ± 0.0	1.27 ± 0.34	100.0 ± 0.0
placenta	0.59 ± 0.11	151.8 ± 65.0	56.57 ± 24.39	4040.3 ± 623.1
heart	0.87 ± 0.23	167.7 ± 21.0	0.27 ± 0.07	23.4 ± 6.0
lungs	0.60 ± 0.11	126.4 ± 16.2	1.67 ± 0.62	120.5 ± 15.1
intestine	0.37 ± 0.13	67.8 ± 13.3	0.52 ± 0.15	33.5 ± 10.2
aortic bodies	4.98 ± 0.33	1188.7 ± 292.4	0.21 ± 0.05	16.9 ± 4.3
adrenals	1.50 ± 0.31	254.0 ± 18.0	0.36 ± 0.06	30.0 ± 2.3
kidneys	0.62 ± 0.10	146.7 ± 34.0	0.50 ± 0.15	41.0 ± 7.8
brain stem	0.18 ± 0.06	35.0 ± 9.8	0.22 ± 0.07	20.4 ± 7.3
hemispheres	0.19 ± 0.00	39.9 ± 5.4	1.06 ± 0.31	79.6 ± 10.8

activity in the liver with the 3H activity in the umbilical vein of the foetus reveals that the 3H concentrations at 5 and 240 min (in nCi/g) did not differ significantly but that the activity in the liver 30 min after the injection, 2.10 ± 0.31 was significantly ($p < 0.001$) higher than the concentration in the umbilical vein and artery, 0.42 ± 0.05 and 0.49 ± 0.11 respectively. The 3H concentration in the kidneys, adrenals, heart, lungs and aortic bodies was also higher than that in the umbilical vein, and that in kidneys and lungs higher than the concentration in the umbilical artery ($p < 0.01$). The 3H activities in the brain, brain stem, intestine and placenta were not significantly different from the umbilical artery or vein values.

Metabolites

Blood 5 min after the injection the concentration of 3HNA in the mother's peripheral blood was significantly higher and that of $^3HMHPG-VMA$ lower than in the umbilical vein of the foetus. The maternal 3HNA concentration was also significantly higher than in the umbilical artery (Table 13).

The $^3HDHPG-DHMA$ activity was higher in the umbilical vein of the foetus than in the maternal vein in five out of six foetuses. The fraction of metabolites travelling to the front was also higher ($p < 0.05$).

TABLE 13. *11N1 and its metabolites in maternal peripheral blood and fetal umbilical veins and arterial blood 5 min after 11N1 administration to the mother. During anaesthesia 1.2 µg or 50 µCi, of 11H NA were injected over 10 sec into the mother's antecubital vein. Samples were taken from maternal peripheral blood, contralateral from the injection, and simultaneously from fetal umbilical venous and arterial blood. NA and metabolite quantities stated in nCi/g (mean ± S.E.M, 6 determinations) and as average percentage of total activity*

Compound	Maternal blood (6)		Umbilical venous blood (9)		Umbilical arterial blood (6)	
	nCi/g	% of total 11H activity	nCi/g	% of total 11H activity	nCi/g	% of total 11H activity
Start	0.09 ± 0.01	12.9	0.06 ± 0.02	6.7	0.05 ± 0.02	9.4
Conjugates	0.13 ± 0.02	18.6	0.07 ± 0.02 ^a	7.8	0.05 ± 0.03 ^a	9.4
NA	0.20 ± 0.03	28.6	0.08 ± 0.01	8.9	0.07 ± 0.02 ^a	13.2
VMN	0.07 ± 0.01	10.0	0.08 ± 0.03	8.9	0.03 ± 0.01	9.4
DHPG-DHNA	0.08 ± 0.01	11.4	0.15 ± 0.03	16.7	0.10 ± 0.04	18.9
MHPG-VMA	0.12 ± 0.01	17.1	0.42 ± 0.10 ^b	46.7	0.17 ± 0.04	32.1
Free	0.01 ± 0.01	1.4	0.04 ± 0.04	4.4	0.04 ± 0.01	7.6

^a $p < 0.02$ difference from maternal value

^b $p < 0.01$

^c $p < 0.005$

in the umbilical vein than in the maternal peripheral blood. The ³H MHPG-VMA concentration in the umbilical vein was higher than in the umbilical artery ($p < 0.02$)

The proportion of conjugated metabolites was higher in the maternal blood than in the umbilical vein or artery ($p < 0.005$). Five min after injection nearly 50 % of the activity behaved on chromatography like ³H MHPG-VMA, exactly as was found in study I.

30 min after the injection the ³H MHPG-VMA concentration was still higher in the umbilical vein and artery than in the maternal peripheral blood ($p < 0.02$) (Table 14). 30 min after the injection the principal metabolite in the umbilical vein and artery was still ³H MHPG-VMA (accounting for about 1/4 of the activity).

The differences in the concentrations of the other metabolites were minimal with no statistically significant differences between the foetal and maternal blood.

After 240 min no statistically significant differences were evident between the concentrations of the various metabolites of ³H NA in the umbilical blood samples and in the maternal peripheral blood (Table 15).

TABLE 14. *HNA and its metabolites in maternal peripheral blood and foetal umbilical venous and arterial blood 30 min after HNA administration to the mother. Samples taken 30 min after the injection. Number of determinations stated in parentheses. See legend to Table 13.*

Compound	Maternal blood (5)		Umbilical venous blood (5)		Umbilical arterial blood (5)	
	nCi/g	% of total H activity	nCi/g	% of total H activity	nCi/g	% of total ³ H activity
Start	0.10 ± 0.02	22.7	0.06 ± 0.01	14.3	0.09 ± 0.03	18.8
Conjugates	0.09 ± 0.02	20.5	0.08 ± 0.02	19.0	0.07 ± 0.02	14.6
NA	0.07 ± 0.02	15.9	0.05 ± 0.01	11.9	0.08 ± 0.02	16.7
NMN	0.03 ± 0.01	6.8	0.03 ± 0.00	7.1	0.03 ± 0.01	6.3
DHPG-DHMA	0.05 ± 0.01	11.4	0.06 ± 0.01	14.3	0.05 ± 0.01	10.4
MHPG-VMA	0.07 ± 0.01	15.9	0.11 ± 0.01	26.2	0.13 ± 0.03 ¹	27.1
Front	0.03 ± 0.01	6.8	0.03 ± 0.01	7.1	0.03 ± 0.01	6.3

¹ < 0.02 difference versus maternal value

TABLE 15. *HNA and its metabolites in maternal peripheral blood and foetal umbilical venous and arterial blood 240 min after HNA administration to the mother. Prior to anaesthesia, 1 HNA was injected into the mother's antecubital vein. Samples were taken 240 min after the injection. Number of determinations in parentheses. See legend to Table 13.*

Compound	Maternal blood (4)		Umbilical venous blood (4)		Umbilical arterial blood (4)	
	nCi/g	% of total H activity	nCi/g	% of total H activity	nCi/g	% of total H activity
Start	0.08 ± 0.01	24.2	0.07 ± 0.01	21.2	0.08 ± 0.03	24.2
Conjugates	0.07 ± 0.02	21.2	0.06 ± 0.01	18.2	0.06 ± 0.01	18.2
NA	0.04 ± 0.00	12.1	0.04 ± 0.02	12.1	0.04 ± 0.01	12.1
NMN	0.02 ± 0.00	6.1	0.02 ± 0.01	6.1	0.02 ± 0.01	6.1
DHPG-DHMA	0.03 ± 0.00	9.1	0.04 ± 0.02	12.1	0.04 ± 0.01	12.1
MHPG-VMA	0.03 ± 0.02	15.2	0.07 ± 0.01	21.2	0.07 ± 0.02	21.2
Front	0.01 ± 0.02	12.1	0.03 ± 0.00	9.1	0.02 ± 0.01	6.1

Foetal tissues and placenta. As in the blood ³H MHPG-VMA was the most abundant metabolite on a percentage basis in all foetal tissues except the lungs, kidneys and adrenals 5 min after the injection. The dominant component in the kidneys and adrenals were metabolites which behaved chromatographically as conjugates (Figs 7–8, Table 16).

However 30 min after injection metabolites behaving as conjugates were also present in high amounts (Figs 8–9, Table 17–18).

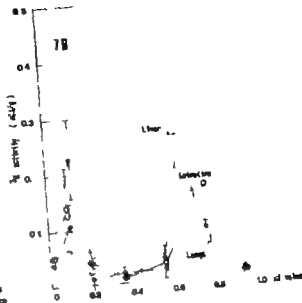
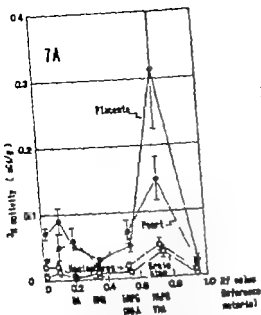


Fig. 7. *H-NA* and its metabolites in fetal tissues 5 min after *H-NA* administration to the mother. Quantity of metabolites in nCi/g. Mean \pm S.E.M. values of 5 to 6 foetuses shown in the figure. — Fig. 7A. Behav. of reference substances on chromatography. See legend to Fig. 5.

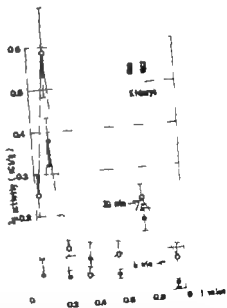
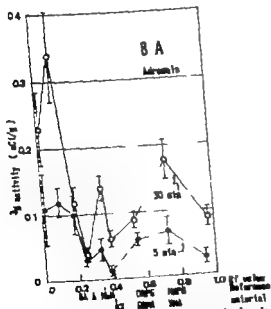


Fig. 8. *H-NA* and its metabolites in fetal adrenals and kidneys 5 and 30 min after *H-NA* administration to the mother. Quantity of metabolites in nCi/g. Mean \pm S.E.M. values for 6 foetuses. Fig. 8A: Distribution of metabolites in adrenals, Fig. 8B: Distribution of metabolites in kidneys. The behaviour of reference material is shown in Fig. 8A. See legend to Fig. 5.

TABLE 16. *Determination of unconjugated and conjugated HVA and its metabolites in the foetal liver 5 min after HVA administration to the mother* Samples taken 5 min after injection of 1.2 μ g or 50 μ Ci of 3 H NA into the mother's antecubital vein. Metabolites determined by paper chromatography without hydrolysis and after acid hydrolysis by heating in 0.4 mol/l perchloric acid in boiling water for 12–15 min. Quantities of NA and metabolites in nCi/g (mean \pm S.E.M) and average percentages of total activity. Number of determinations in parentheses.

Compound	Without hydrolysis (5)		After acid hydrolysis (6)	
	nCi/g	% of total 3 H activity	nCi/g	% of total H activity
Start	0.05 ± 0.01	5.7	0.03 ± 0.00	3.4
Conjugates	0.23 ± 0.07	26.4	0.00 ± 0.01	6.7
NA	0.04 ± 0.01	4.6	0.19 ± 0.04	21.3
NAEN	0.01 ± 0.00	1.1	0.11 ± 0.01	12.4
DHPG-DHMA	0.04 ± 0.00	4.6	0.04 ± 0.01	4.3
MHPG-VMA	0.49 ± 0.11	56.3	0.44 ± 0.09	49.4
Front	0.01 ± 0.00	1.1	0.02 ± 0.00	2.2

$p < 0.02$ difference from value without hydrolysis

$p < 0.01$

$p < 0.005$

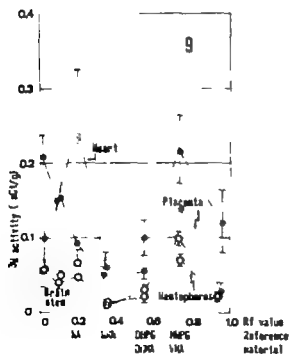


Fig. 9. 3 H NA and its metabolites in foetal tissues 30 min after 3 H NA administration to the mother. 1.2 μ g or 50 μ Ci of 3 H NA injected over 10 sec into the mother's antecubital vein. Foetal tissue samples taken 30 min after the injection. NA and metabolite quantities in nCi/g mean \pm S.E.M for 5 to 11 foetuses.

TABLE 17. *Unconjugated and conjugated H V1 and its metabolites in foetal lung and intestine 30 min after H V1 administration to the mother. Samples taken 30 min after the injection. See legend to Table 16.*

Compound	Lungs (5)			Intestine (4)			
	Without hydrolysis		After acid hydrolysis	Without hydrolysis		After acid hydrolysis	of total H activity
	nCi/g	% of total H activity		nCi/g	% of total H activity		
Start	0.24 ± 0.06	23.5	0.03 ± 0.01	0.11 ± 0.03	21.2	0.04 ± 0.01	7.5
Conjugates	0.45 ± 0.04	47.9	0.07 ± 0.01	0.14 ± 0.05	26.9	0.04 ± 0.01	7.5
NA	0.06 ± 0.01	6.4	0.10 ± 0.03	0.07 ± 0.02	13.5	0.07 ± 0.01	13.2
NAMN	0.02 ± 0.01	2.1	0.05 ± 0.01	0.02 ± 0.00	3.8	0.05 ± 0.01	9.4
DIPEG-DHMA	0.03 ± 0.01	3.2	0.06 ± 0.01	0.03 ± 0.01	5.8	0.03 ± 0.01	9.4
ΔDIPEG-VMA	0.11 ± 0.01	11.7	0.54 ± 0.04	0.12 ± 0.02	23.1	0.21 ± 0.05	39.6
Total	0.03 ± 0.01	3.2	0.10 ± 0.02	0.03 ± 0.01	5.8	0.07 ± 0.07	13.2

$p < 0.05$ difference π vs π without hydrolysis

$p < 0.01$

$p < 0.005$

TABLE 18. *Determination of conjugated H-NA metabolites in foetal liver 30 min after H-NA administration to the mother* Samples taken 30 min after injection of 1.2 μ g, or 50 μ Ci, of 1 H-NA into the mother's antecubital vein. Metabolites determined before hydrolysis and after both acid and enzyme hydrolyses. Acid hydrolysis was by heating for 12–15 min in 0.4 mol/l perchloric acid in boiling water. Enzyme hydrolysis was with a sulphatase + β -glucuronidase preparation (Gluculase^R) for 24 hrs at 37°C. Quantity of metabolites stated in nCi/g (mean \pm S.E.M) and a average percentage of total activity. Number of determinations in parentheses.

Compound	Without hydrolysis		After acid hydrolysis		After sulphatase + β -glucuronidase hydrolysis	
	(5)		(5)		(5)	
	nCi/g	of total H activity	nCi/g	% of total H activity	nCi/g	% of total H activity
Start	0.29 \pm 0.03	15.9	0.03 \pm 0.01	1.7	0.04 \pm 0.01	2.2
Conjugates	0.31 \pm 0.03	17.0	0.06 \pm 0.02 ^a	3.3	0.07 \pm 0.02 ^a	3.8
NA	0.09 \pm 0.01	4.9	0.09 \pm 0.02	5.0	0.03 \pm 0.01	1.6
NaN	0.05 \pm 0.01	2.7	0.33 \pm 0.03 ^a	18.3	0.32 \pm 0.04 ^a	17.6
DHPG-DHMA	0.08 \pm 0.01	4.4	0.04 \pm 0.01	2.2	0.02 \pm 0.01	1.1
MHPG-VMA	0.93 \pm 0.08	51.1	1.17 \pm 0.08 ^a	63.0	1.29 \pm 0.08 ^a	70.9
Front	0.07 \pm 0.02	3.8	0.08 \pm 0.02	4.4	0.03 \pm 0.03	2.7

^a $p < 0.05$ difference versus value without hydrolysis

^b $p < 0.01$

^c $p < 0.001$

The sample quantities from the aortic bodies were so small that the metabolites could only be analysed in a part of them. In these cases descending chromatography was used. The separation of NVA and NVN/MN was fairly good though not as complete as that of the different metabolites of NA. It is observed that ³H A and ³H VN were not positively demonstrable in the aortic bodies or in the adrenals.

240 min after the injection a large proportion of the metabolites behaved as conjugates. In this connection only the distribution of metabolites from the heart, liver, intestine and brain stem are presented (Fig. 10).

Conjugates

In the case of the 5 min samples, acid hydrolysis and separation of conjugated metabolites was only carried out with liver lung and intestinal homogenates, and only if there was sufficient material for several runs (Table 16)

All these determinations yielded similar results 5 min after the injection ^3H NA and ^3H NMN could be found in the foetus in conjugated form.

30 min after the injection, ^3H NMN and part of the ^3H MHPG VMA were also present in the same tissues in conjugated form. This was established using both acid hydrolysis and enzymic hydrolysis with Glucuronase² which contains both sulphatase and β -glucuronidase (Tables 17-18)

The umbilical blood samples and the samples from the foetal adrenals, kidneys and paraganglia were not large enough for conjugate determinations. Conjugates were not analysed in the samples taken 210 min after the injection. The quantities of amniotic fluid and urine collected and the ^3H activity in them were too small to permit any statement concerning their metabolite content.

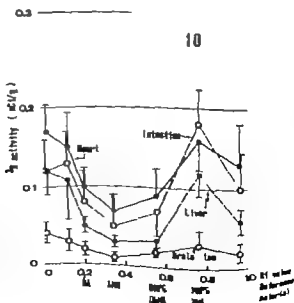


Fig. 10 HNA and its metabolites in foetal tissues 240 min after HNA administration to the mother. Foetal tissue samples taken 240 min after the injection. NA and metabolite quantities $1 \mu\text{Ci/g}$ mean \pm S.E.M. of 4 foetuses. For other particulars, see legend to Fig. 9

Comment

All the ^3H NA metabolites were found in the foetal umbilical blood 5, 30 and 240 min after injection. The ^3H MHPG-VMA level was higher in the foetal than in the maternal blood therefore ^3H NA is metabolized in the placenta and/or ^3H MHPG VMA cannot pass freely from the foetus to the mother.

^3H NA metabolites accounted for the major part of the activity in the foetal tissues. In view of the minimal amount of intact ^3H NA that can pass from the mother into the foetus, the ^3H NA uptake capacity of the foetal tissues was not determined in these experiments.

The placenta metabolizes ^3H NA, and the foetus obviously continues this metabolic action with the aid of both MAO and COMT. The foetus also has capacity to conjugate these metabolites. In the placenta no p-nitrophenol-sulphating activity (Pulkkinen 1963) nor any activity of the enzymes participating in the glucuronic acid conjugation of o-aminophenol (Hartnala and Pulkkinen 1955; Dutton 1959) could be detected. It is therefore probable that no conjugation of catecholamines occurs in the placenta. However the present study does not exclude the possibility that metabolites conjugated in the mother might pass into the foetus.

III Metabolism of ^3H noradrenaline in the foetoplacental unit

Material and methods

In this part of the study a total of 13 foetuses weighing from 15 to 274 g were investigated. dl- ^3H NA 25–85 $\mu\text{Ci/kg}$ or 0.6 to 2.1 $\mu\text{g/kg}$ was injected into the umbilical vein of eight foetuses (20–274 g) over a period of 30 seconds. Efforts were made to keep the dose as near to 50 $\mu\text{Ci/kg}$ as possible. In order to estimate its weight the foetus was removed from the uterus in every case. dl- ^3H NA was injected into the jugular vein of three other foetuses after dissection and at a later stage ^3H NA was injected into the jugular vein of two further foetuses, 53 and 24 $\mu\text{Ci/kg}$ or 1.0 and 0.45 $\mu\text{g/kg}$ respectively. The foetoplacental circulation was maintained intact for 15 min. The umbilical cord was then closed and the foetus and placenta placed in separate containers. One hour after the start of the injection, the tissues were excised in the same order as in the preceding studies: heart, lungs, liver, intestine, adrenals, kidneys, brain and brain stem.

The metabolites were determined in a part of the tissues, only the weighed tissue was placed in 0.4 mol/l perchloric acid, continuing thereafter as stated in the chapter on methods. Part of the tissues were dried at -4°C and their total activity determined by combustion.

The mother's premedication regimen consisted of the following: atropine 0.1 mg/10 kg, pethidine 50–100 mg and promethazine 25 mg i.m. one hour before the operation. Induction was carried out with thiomebumal, and the anaesthesia was continued with a mixture of N₂O/O₂ and, for relaxation of the uterus, halothane at a concentration of 0.5 % until the foetus and placenta had been detached and the umbilical circulation closed.

The metabolites were determined in the same manner as in the preceding studies, except that the whole of the activity running below NA was determined as one single strip and that running above VMA was not determined.

Results

Total activity

The activity in the tissues, in nCi/g of tissue, and their content per tissue are shown in Table 19. It can be seen that the ³H activity is remarkably uniformly distributed between the different tissues, and that the mode of injection had some influence on the distribution. The proportion taken up by the liver decreased ($p < 0.01$) and that by the placenta increased when the injection was made into the jugular vein. The ³H activity (in nCi/g) in the brain and brain stem was very similar but lower on the average than in the other tissues ($p < 0.001$ – 0.01). No statistically significant differences were apparent between any of the other tissues.

The ³H content of the placenta (nCi) was highest, followed by the liver and the lungs.

All data showed a considerable dispersion, and the dispersion was also rather high in the amount of ³H activity injected. The ³H activity in the tissues examined amounted to 10–15 % of the quantity injected. Blood samples were drawn from three mothers 15 min after the injection, and it could be seen that some activity passes from the foetus into the mother. However the amount was so small (0.08 nCi/ml) that metabolite determination was not possible.

The foetuses injected with 1-³H NA were examined at a later time, in 1972, and are therefore considered separately. On account of the small subject number no definite conclusions can be drawn, but it appears possible that the 1-³H NA uptake by the foetal tissues is higher than that of the dl-form, or that it is metabolized to a lesser degree (Table 20).

The activity in the aortic bodies was highest, and that in the brain lowest. Otherwise the distribution was fairly uniform.

TABLE 19 *Distribution of H activity in placenta and foetal tissues after administration of $1\text{ }H\text{ }Yl$ into umbilical or jugular vein of foetus in the foetoplacental unit. Mean \pm S.E.M of 3 (administration to jugular vein) to 7-8 determinations (administration to umbilical vein)*

Tissue	H concentration, nCi/g	% of 3H concentration in the liver	Total 3H content/tissue nCi	% of H content in the liver
<i>3H-NA administration into the umbilical vein</i>				
liver	25.8 ± 4.4	100.0 ± 0.0	108.7 ± 32.7	100.0 ± 0.0
placenta	1.5 ± 0.4	7.6 ± 2.3	165.7 ± 51.2	299.0 ± 138.0
heart	13.7 ± 0.4	72.3 ± 17.1	8.2 ± 2.7	11.3 ± 3.0
lungs	18.1 ± 3.4	89.1 ± 23.2	63.8 ± 27.9	113.4 ± 36.1
intestine	10.8 ± 2.7	74.4 ± 30.5	20.3 ± 4.0	68.7 ± 24.3
adrenals	22.8 ± 5.0	97.5 ± 23.1	10.1 ± 3.7	12.2 ± 3.3
kidneys	14.3 ± 3.8	68.7 ± 13.8	16.3 ± 8.6	16.2 ± 5.1
brain stem	2.8 ± 0.7	11.7 ± 3.9	3.8 ± 1.0	9.0 ± 4.2
hemispheres	2.9 ± 0.8	16.2 ± 6.4	27.9 ± 11.5	54.0 ± 18.6
<i>3H-NA administration into the jugular vein</i>				
liver	4.4 ± 1.0	100.0 ± 0.0	11.9 ± 5.3	100.0 ± 0.0
placenta	7.4 ± 3.1	148.4 ± 16.5	747.9 ± 384.9	4222.8 ± 1621.3
heart	18.2 ± 8.2	507.8 ± 239.7	4.6 ± 2.1	67.3 ± 32.8
lungs	5.0 ± 1.0	107.8 ± 13.8	17.7 ± 5.0	107.6 ± 0.0
intestine	7.0 ± 0.2	174.7 ± 37.5	10.7 ± 4.0	93.0 ± 17.2
adrenals	18.0 ± 12.5	513.7 ± 348.2	2.3 ± 0.5	44.6 ± 31.4
kidneys	16.3 ± 6.8	417.7 ± 203.2	5.4 ± 1.3	65.4 ± 26.0
brain stem	1.4 ± 0.0	33.2 ± 12.5	2.5 ± 0.1	16.3 ± 4.9
hemispheres	1.0 ± 1.3	32.1 ± 42.4	16.4 ± 10.6	124.6 ± 92.4

TABLE 20 *Concentration and content of 3H in placenta and foetal tissues after administration of $1\text{ }H\text{ }Yl$ into jugular vein of foetus in the foetoplacental unit. Individual values of 14 foetuses; weight of foetuses in parentheses.*

Tissue	H concentration, nCi/g		Total H content/tissue nCi	
	(94 g)	(253 g)	(94 g)	(253 g)
liver	43.3	21.0	160.8	221.7
placenta	0.8	8.0	93.2	1031.9
heart	129.9	92.0	57.2	135.1
lungs	137.6	23.3	590.1	173.1
intestine	32.7	23.6	68.3	116.4
sorotic bodies	321.8	158.9	8.1	7.8
adrenals	16.7	34.5	56.2	20.1
kidney	41.1	31.6	42.6	91.4
brain stem	4.6	1.1	8.5	2.6
hemispheres	2.4	1.0	4.3	31.6

Metabolites

Since the ^3H NA dosage per unit weight was highly variable, the metabolite levels are considered on the basis of their relative (percentage) distribution. dl ^3H NA accounted for the greatest part of the activity in the heart, intestine, kidneys and adrenals, on the average 40–55 % of the total (Figs 11–12). In the adrenal samples NA and A were

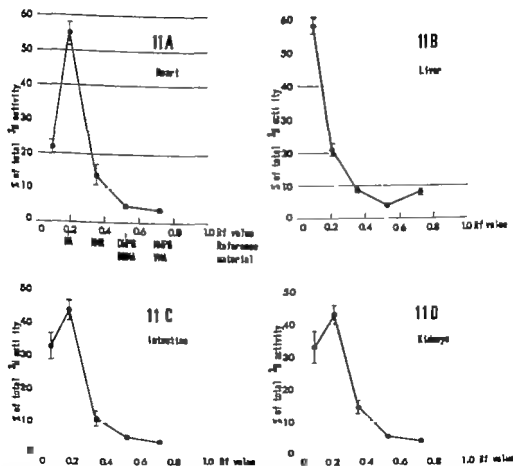


Fig. 11 Metabolism of ^3H NA in the foetoplacental unit. 0.6 to 2.1 $\mu\text{g}/\text{kg}$ of dl- ^3H -NA injected into the foetoplacental unit through the umbilical vein or jugular vein of the foetus. Umbilical cord clamped after 15 min. Metabolite quantities given as percentage of total activity. The results are mean \pm S.E.M. values for 6 to 8 foetuses. — Fig. 11A: Foetal heart, Fig. 11B: Foetal liver, Fig. 11C: Foetal intestine, Fig. 11D: Foetal kidneys. Fig. 11A shows the behaviour of the reference substances on chromatography.

not separated. In the remainder of the tissues the major part of the activity behaved on chromatography as conjugated more than 55 % in the liver and on average, 40 % in the lungs. The proportion of conjugate like activity was lowest in the heart, about 22 % and in the other tissues about 30–35 %.

A very similar per cent distribution was observed in the two foetuses injected with $1\text{ }^3\text{H NA}$. However the proportion of $^3\text{H NA}$ in the heart intestine and kidneys was higher than in the foetuses which were injected with $\text{dl } ^3\text{H NA}$ (Figs 13–15). The $1\text{ }^3\text{H NA}$ metabolite distribution in the blood and brain of these foetuses were very similar. Approximately half of the substance administered behaved as NA on chromatography.

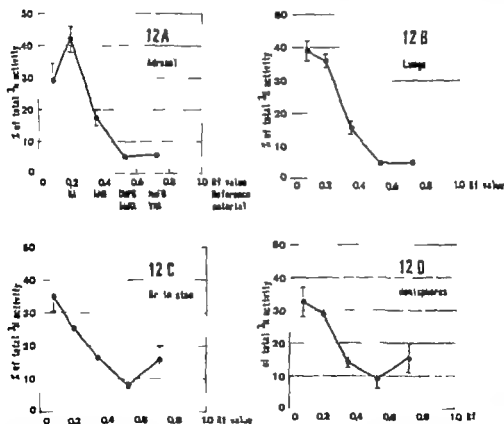


Fig. 12. Metabolism of $^3\text{H NA}$ in the foetal/placental unit. Fig. 12A: Metabolites in the μ adrenals, Fig. 12B: in foetal lungs, Fig. 12C: in foetal brain stem, Fig. 12D: in foetal gonads. Mean \pm S.E.M. values for 6 to 8 foetuses. For other particulars see legend to Fig. 11.

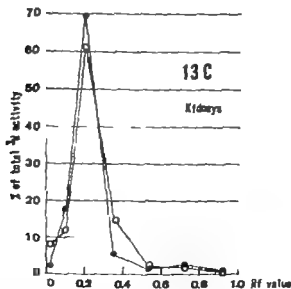
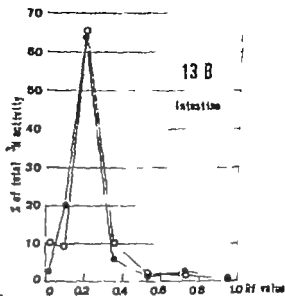
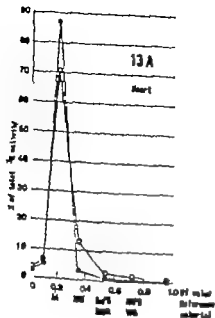


Fig. 13. Metabolism of H-NA in the foetoplacental unit. 0.45 $\mu\text{g/kg}$ of L-H-NA administered to one foetus, and 1.0 $\mu\text{g/kg}$ to another by 1 injection into the jugular vein. Umbilical cord blocked after 15 min. Results stated as percentages of total activity — Fig. 13A. Metabolites in foetal heart, Fig. 13B; foetal intestine, Fig. 13C; in foetal kidneys. Fig. 13A shows the behaviour of reference material on chromatography. \circ Foetus weighing 233 g. \bullet Foetus weighing 94 g.

Of the metabolites of dl ^3H NA and l ^3H NA, more NMN than DHPG-DHMA was found, and usually equal amounts of MHPG-VMA and DHPG DHMA. In the paraganglia and adrenals of two foetuses no l ^3H A or l ^3H MN could be detected (Fig 15) The conjugates were also determined, after acid hydrolysis and enzymic hydrolysis, in the liver supernatant of the foetuses injected with l ^3H NA. It could be clearly seen that in both foetuses ^3H NA and ^3H NMN were present in the liver conjugated to sulphate (Fig 16)

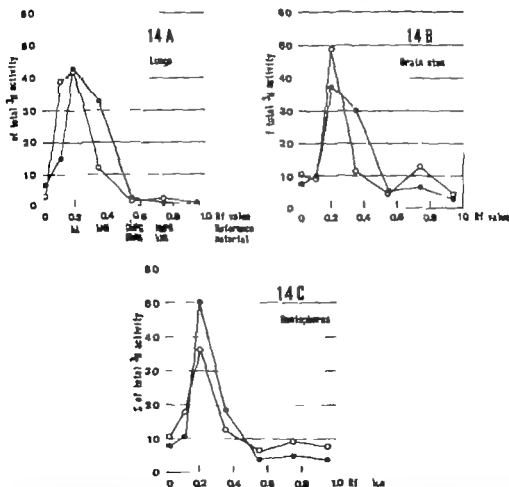


Fig. 14 Metabolism of ^3H NA in the foetoplacental unit. Fig. 14A. Metabolites in foetal lungs. Fig. 14B. in foetal brain stem Fig 14C. foetal hemispheres. Fig. 14A show the behaviour of reference compounds on brainography. For other particulars, see legend: Fig. 13.

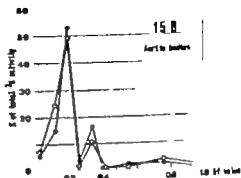
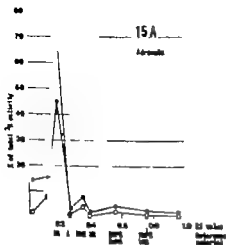


Fig. 15. Metabolism of HNA in the foetal placental unit. Fig. 15A: Metabolism adrenalis, Fig. 15B: Metabolism in aortic bodies. For other particulars, see legend to Fig. 15.

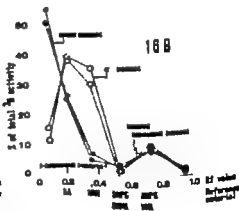
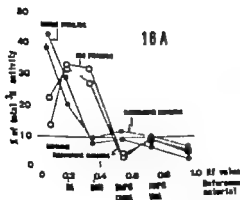


Fig. 16. Conjugates of HNA and its metabolism in the foetal liver in the foetal placental unit 1. The foetal placental unit 1 HNA was injected into the jugular ei of two foetuses, 0.45 $\mu\text{g/kg}$ into one and 1.0 $\mu\text{g/kg}$ into the other. The umbilical cord was blocked after 15 min. Metabolites determined before hydrolysis and after acid as well as enzymic hydrolysis. Acid hydrolysis was performed by heating the supernatant, prior to chromatography for 12 min in boiling water in 0.4 mol/l perchloric acid, enzymic hydrolysis using an enzyme preparation containing sulphatase and β -glucuronidase (Glumase[®]) or preparation containing β -glucuronidase (Metodase[®]) for 24 hrs at 37°C. Metabolic quantities as percentages of total activity. Fig. 16A: Foetus weighing 94 g, Fig. 16B: Foetus weighing 233 g.

Comment

The experimental design resulted in foetal asphyxia the degree of which was not measured. However the heart functioned in all foetuses after 15 min. It is clear from this study that the foetus is well able to metabolize ^3H NA. MAO, COMT and sulphate conjugating activity was detected.

The heart, intestine, kidneys and adrenals of the foetus can obviously actively remove ^3H NA from the circulating blood judging from the remarkable proportion of ^3H NA in them, nearly 90 % in the heart of one foetus injected with 1 ^3H NA. The brain contained a distinctly measurable quantity of ^3H although the amount of ^3H NA was only about 1/3 of the total. The blood/brain barrier is clearly not yet fully developed in a foetus of this age. The tissue concentrations in the foetuses injected with 1 ^3H NA were fairly high which is partly due to the fact that the material was injected into the jugular vein, the dissection of which is rather time-consuming. As a consequence, the placental circulation has time to deteriorate, and the leakage of ^3H activity into the mother becomes less.

IV Distribution and metabolism of ^3H noradrenaline in the human foetus after removal from the uterus

Material and methods

The material for this study comprised six foetuses aged 13 to 20 weeks weighing between 35 and 260 g. The foetuses were obtained at the interruption of pregnancy performed by the laparotomy approach.

After removal of the foetus and placenta from the uterus, the umbilical cord was closed within 1 min and 1 ^3H NA (specific activity 8.7 Ci/mmol) $0.11 \pm 0.01 \mu\text{Ci}/100 \text{ g}$ or ca. $0.02 \mu\text{g}/\text{kg}$ was injected over a period of 10 seconds into the umbilical stump. In view of the volume error the umbilical cord was not flushed, instead it was "milked" towards the foetus. The foetus was transferred within 2 min from the operating theatre in a covered cardboard box, to a heating bath at 37°C, where it was left for 15 min. During and all through the last minute the foetal heart rate was counted. A blood sample was then taken by making an opening in the right atrial wall of the heart with a scissors and drawing the blood from the cardiac sac with a pipette. This procedure took 1–2 minutes. The blood was directly pipetted into 0.4 mol/l perchloric acid, at 0°C on an ice bath. The heart then was removed. A piece of the liver was also immersed, at this time in 0.4 mol/l perchloric acid, and rapidly cooled to -15 – -20 °C. The other tissues were excised, weighed and dried for a few days at $+4$ °C. The metabolites and conjugates in the blood, liver and urine of the foetus were analysed using the method presented in the chapter on methods. In the rest of the tissues the tissue activity was determined by combustion of the total organ or part thereof.

Results

After 15 min, the heart rate of the foetus averaged 75 ± 11 beats per min (mean \pm S.D.) In one foetus the following Astrup values were obtained. pH 6.94 $p\text{CO}_2$ 78.6 mmHg and BE -15.6 meq/l. This particular foetus had a heart rate of 62 beats per min during the last minute.

Total activity

The ^3H activity in the tissues is given in Table 21. The activity in the aortic bodies, averaging 8.18 ± 0.59 nCi/g, was higher than in any of the other foetal tissues ($p < 0.001$).

There were no statistically significant differences in the total activity in the heart, liver and adrenals. The activity in the liver 3.93 nCi/g, was higher than that in the lungs ($p < 0.005$) intestine ($p < 0.005$) kidneys and brain and brain stem. The activity in the brain and brain stem (0.15 and 0.16 nCi/g) was significantly lower than in all other tissues, including the skin of the head ($0.01 > p < 0.001$).

The activity in the blood, urine and skin was almost the same, 1.08 ± 0.17 , 0.96 ± 0.28 and 0.96 ± 0.08 nCi/g, respectively ($p > 0.05$).

The total ^3H content, calculated per tissue, was significantly greater in the liver ($p < 0.001$) than in all the other tissues. Of the remainder

TABLE 21. Concentration and content of ^3H in foetal tissues after administration of ^3H -NA into maternal vein after clamping umbilical cord. Mean \pm S.E.M. of 6 determinations.

Tissue	^3H concentration, nCi/g	% of ^3H concentration in the liver	Total ^3H content/tissue, nCi	% of ^3H content in the liver
liver	3.93 ± 0.49	100.0 ± 0.0	21.99 ± 7.71	100.0 ± 0.0
heart	3.44 ± 0.41	98.2 ± 19.9	2.28 ± 0.80	10.3 ± 1.8
lungs	1.14 ± 0.19	31.5 ± 5.8	7.01 ± 2.71	27.3 ± 5.5
intestine	1.38 ± 0.28	33.7 ± 5.3	4.77 ± 1.80	22.1 ± 3.8
aortic bodies	8.18 ± 0.59	224.5 ± 29.5	0.12 ± 0.01	1.3 ± 0.4
adrenals	2.77 ± 0.28	78.0 ± 16.9	1.33 ± 0.36	6.4 ± 0.6
kidneys	1.89 ± 0.32	40.4 ± 6.1	1.83 ± 0.42	10.2 ± 2.0
brain stem	0.15 ± 0.00	4.2 ± 0.7	0.35 ± 0.10	1.8 ± 0.4
hemispheres	0.16 ± 0.00	4.5 ± 1.0	2.53 ± 0.67	11.7 ± 1.9
skin of the head	0.96 ± 0.08	28.9 ± 4.3		

the lungs and intestine contained the highest activity. The heart and brain contained almost equal amounts, 2.28 ± 0.80 and 2.53 ± 0.87 nCi, respectively. The activity was lowest in the para aortic ganglia, 0.12 ± 0.01 nCi (Table 21).

The activity contained in the tissues taken as samples accounted (on the average) for $27.7 \pm 2.9\%$ of the radioactive substance injected. This value is quite consistent with the proportion of the total foetal weight in the samples analyzed which was 25.3% .

Metabolites

If the average metabolite distribution at a given time in the liver, blood and urine (Table 22) are compared, it can be seen that the metabolite pattern in the blood and urine is almost identical. On the basis of this evidence, under these experimental conditions about half of the ^3H NA metabolites would be excreted in unchanged form in the urine and the end product MHPG-VMA would only account for about 7% of the total. On the other hand the proportion of conjugated metabolites in the liver was much higher than in the blood and urine. The same is true of the ^3H DHPG-DHMA and ^3H MHPG-VMA concentrations ($0.01 > p < 0.001$). It was clear from the results that the foetus can metabolize injected NA independent of the placenta, through the action of both MAO and COMT.

TABLE 22. Fate of ^3H -NA in the foetus outside the uterus. $0.02 \mu\text{g/kg}$ or $1.1 \mu\text{Ci/kg}$, of ^3H NA was injected into the umbilical vein of the human foetus after clamping the umbilical cord. Samples were taken 15 min after the injection. Quantity of NA and metabolites in nCi/g (mean \pm S.E.M., 4–6 determinations) and average percentages of metabolites from total activity (number of determinations in parentheses).

Compound	Liver (6)		Blood (6)		Urine (4)	
	nCi/g	% of total H activity	Ci/g	% of total ^3H activity	nCi/g	% of total H activity
Start	0.56 ± 0.14	14.9	0.06 ± 0.01	5.6	0.12 ± 0.07	13.2
Conjugates	1.55	0.32	0.17 ± 0.03	15.7	0.09 ± 0.04^1	9.9
NA	0.74	0.08	0.41 ± 0.11	40.7	0.46 ± 0.18	50.5
VMA	0.23	0.02	0.14 ± 0.03	13.0	0.07 ± 0.02	7.7
DHPG-DHMA	0.22	0.03	0.10 ± 0.02	9.3	0.07 ± 0.02	7.7
MHPG-VMA	0.38	0.03	0.09 ± 0.01^2	8.3	0.06 ± 0.02^2	6.6
Free	0.03	0.02	0.08 ± 0.02	7.4	0.01 ± 0.01	1.1

$p < 0.01$ difference from value in the liver

$p < 0.005$

Conjugates

The conjugated metabolites were analysed both after acid and enzymic hydrolysis, in order to determine the mode of conjugation

The chromatogram obtained without preceding hydrolysis is clearly different from those after acid and Glusulase[®] hydrolysis (Table 23). The activity in the conjugate region was reduced by acid hydrolysis ($p < 0.01$) the proportion of NA correspondingly increased from 0.74 ± 0.08 to 1.23 ± 0.16 ($p < 0.02$) and that of NMN from 0.23 ± 0.02 to 1.28 ± 0.24 nCi/g ($p < 0.01$). The fraction running above VMA increased from 0.08 ± 0.02 to 0.16 ± 0.03 ($p < 0.02$).

After treatment with both β -glucuronidase and sulphatase the fraction remaining at the start did not differ significantly from the unhydrolysed value. On the other hand the fraction running up over the start, 0.33 ± 0.05 nCi/g, was significantly ($p < 0.01$) lower than in the absence of hydrolysis. The NA ($p < 0.02$) and NMN ($p < 0.01$) levels were also significantly increased.

β -glucuronidase treatment did not alter the percentage distribution of the metabolites. Only the activity in the fraction running ahead of VMA was seen to increase from 0.08 ± 0.02 to 0.26 ± 0.04 nCi/g ($p < 0.01$).

TABLE 23. Configuration of 11 and its metabolites in the serial liver outside the uterus. Metabolites determined before hydrolysis and after acid and enzyme hydrolysis. Acid hydrolysis was by heating for 12 min in 0.4 mol/l perchloric acid in boiling water and enzyme hydrolysis by means of sulphatase + β -glucuronidase preparation (Gibbs and Gibb) and of β -glucuronidase preparation (Koch and Gibb) for 24 hrs at 37°C. Metabolite quantities in nCi/g (mean \pm S.E.M.) to determinations and average percentages from total activity. For other particulars, see legend to Table 22.

Compound	Without hydrolysis		After acid hydrolysis		After sulphatase + β -glucuronidase hydrolysis		After β -glucuronidase hydrolysis	
	nCi/g	% of total H activity	Ci/g	% of total H activity	nCi/g	% of total H activity	nCi/g	% of total H activity
Start	0.56 \pm 0.14	14.9	0.10 \pm 0.04	2.7	0.53 \pm 0.05	8.8	0.56 \pm 0.10	14.7
Conjugates	1.55 \pm 0.52	41.2	0.26 \pm 0.04	7.0	0.53 \pm 0.05	8.8	1.26 \pm 0.22	33.2
VA	0.74 \pm 0.08	19.7	1.23 \pm 0.16	33.3	1.19 \pm 0.15	31.7	0.87 \pm 0.16	22.9
VA-N	0.23 \pm 0.02	6.1	1.28 \pm 0.24	34.5	1.06 \pm 0.19	28.3	0.22 \pm 0.03	5.8
DIHP-DIHA	0.22 \pm 0.03	5.9	0.27 \pm 0.03	7.3	0.23 \pm 0.06	6.7	0.23 \pm 0.03	6.6
DIHPG-VAHA	0.38 \pm 0.05	10.1	0.41 \pm 0.05	11.1	0.41 \pm 0.05	10.9	0.38 \pm 0.05	10.0
Free	0.08 \pm 0.02	2.1	0.16 \pm 0.03	4.3	0.18 \pm 0.04	4.8	0.26 \pm 0.04	6.8

$P < 0.02$ difference versus value without hydrolysis

$P < 0.01$

Comment

In this study distinct asphyxia of the foetus prevailed. This was ascertained only on the basis of the heart rate but was confirmed in one foetus by an Astrup analysis. However the heart was distinctly active 15 min after the injection in all foetuses. The foetal liver was the most important tissue in NA inactivation. The greater part of the activity was discovered in the liver some 14 % of the quantity injected although the weight of the liver accounts for only 4 % of the total foetal weight, on the average. This situation probably arises on account of the route of injection. Another factor which may contribute is the large quantity of blood received by the liver when the umbilical circulation is closed.

The quantity of metabolites in the liver was considerable, and the most significant mode of inactivation appeared to be conjugation with sulphuric acid. Under these experimental conditions the oxidative metabolic pathway mediated by MAO is obviously impaired owing to the prevailing asphyxia. Because of this the products of such oxidative metabolism DHPG, DHMA, MHPG and VMA were only found in trace amounts, even in the liver. Under these conditions the metabolism seems to take place mainly *via* COMT action and *via* sulphate conjugate formation.

DISCUSSION

Paper chromatography and oxidation method

In the method employed in this study for the determination of ^3H NA and its metabolites after separation by paper chromatography Kaartinen's sample oxidizer (1969) was used. It would also have been possible to determine the radioactivity in each fraction after chromatography by placing the appropriate stained or unstained sections directly in the scintillation liquid (see Goodall *et al.* 1964; Schanberg *et al.* 1968b). This would simplify the method, but the counting efficiency would be inferior. Separation by paper chromatography was combined with combustion because thereby pure, colourless samples and a good counting efficiency were obtained and because paper is a most suitable material for burning. In Kaartinen's apparatus the standard deviation of the results is low, 4 dpm, and the recovery is high, ca 99%. This makes the apparatus eminently suitable for the measurement of small amounts of radioactivity.

The method employed is simple and easy to execute. It can thus be used for the analysis of large numbers of samples. The chromatographic separation of NA and of its metabolites achieved was well defined and the various compounds were located by staining prior to the combustion. In this method all metabolites are simultaneously carried through the same process; as a result, activity is not carried into different fractions and the recoveries of all compounds were good, between 94.1 and 97.1%, and showed no statistically significant variation. It was shown that of any given radioactive metabolite only 0.5–1.2% could be recovered after chromatography in another metabolite fraction. The reproducibility of the method is good; present results reveal that the percentage of the total activity in the individual metabolite fractions was constant in seven parallel analyses of the same liver extract. The coefficient of variation was greatest for the metabolites with the lowest activities, from 5 to 24% according to the activity.

Running time for paper chromatography is longer than for thin layer chromatography and the method is made even more time-consuming by the long equilibration period. However thin layer chromatographic procedures are less suitable for combining with a combustion system. In the cellulose thin layer chromatographic method of Giese *et al.* (1967) which resembles the present method only marginal areas 1 cm in width are stained, which might lead to some confusion in metabolite identification. On the cellulose plates used by Giese *et al.* (1967) an aliquot of 0.2 ml only can be accommodated which restricts the possibilities of measuring low ^3H activities.

The paper chromatographic system described is not particularly suitable for separating DHPG from DHMA, MHPG from VMA, NA from A and NAM from AN. It should also be noted that formic acid is rather rapidly esterified to butyl formate in butanol thus the chromatographic solvent is somewhat unstable and could result in variable chromatographic behaviour of samples. However the separation achieved was good and constant throughout the study and no confusion between metabolites could occur since the paper was always stained prior to combustion.

The method of Rutschmann *et al.* (1965) involved a similar combination of paper chromatography and combustion. It could obviously be applied to the separation of NA and its metabolites. It is, however slower than the present method and involves a greater number of steps and would have proved cumbersome for handling large numbers of samples. It was therefore considered unsuitable for the present study.

The method of Kopin *et al.* (1961) or its modifications are the most widely used for the determination of radioactive tracer labelled NA and of its metabolites (*e.g.* Wurtman, Kopin and Axelrod 1963, Glowinski and Iversen 1966, Landsberg and Axelrod 1968, Fauman, Meyers and Schowen 1973). In these methods, the metabolites are separated into numerous groups by a number of column chromatographic procedures and the losses of ^3H activity in the different fractions vary considerably. A method involving as many steps as this is not of particular use when the number of samples to be analysed is great, and the large variation in recovery is objectionable especially if the ^3H activity levels are low.

Influence of asphyxia on the results

In order to maintain the umbilical circulation, fluothane was administered. Such treatment relaxes the uterus. The heart of all the foetuses was active throughout the experimental period. The influence of exteriorization of the human foetus on its circulation has been studied in this clinic (Rudolph *et al.* 1971) and the experimental arrangement used in the present study was based on their findings. They showed that the blood gas analyses of the umbilical vessels were nearly constant for 15 min that during this time, owing to asphyxia, the placental circulation is reduced to about 50 % of its original volume and this results in a redistribution of the circulation in the foetus. The proportion of the cardiac output received by the brain, adrenals and myocardium increases, while that of the lungs, intestine and kidneys decreases.

In an effort to estimate the way in which the potential redistribution of the circulation may affect present results one could imagine that in the foetus remaining *in utero* (experimental arrangement II) there will be no asphyxia. In the foetoplacental unit (experimental arrangement III) a fairly strong asphyxic situation has already obviously developed during the test, and when the foetus was kept outside the uterus with its umbilical cord clamped (experimental arrangement IV) an anoxic state prevailed. Judging by the ^3H content of the foetal tissues the results are consistent with the idea that in an asphyxic situation the quantity of blood going to the heart, at least, would increase, whereas no increase in the blood flow to the brain and adrenals has been positively observed.

However the behaviour of NA and its metabolites was basically the same in the foetoplacental unit, in the acute foetal sampling experiments carried out 1 to 5 min after injection of ^3H NA into the mother in the foetus *in utero* and in the absence of asphyxia. It has thus been possible to solve the problems outlined in the beginning of this report. The investigation of the ability of the foetus to conjugate ^3H NA and its metabolites after removal from the uterus was qualitative by its nature and anoxia was no impediment in the assessment of the results.

It is difficult to design experiments on human subjects that do not involve the added parameter of drug effects. General anaesthesia does not appear to have any effect on the rate of disappearance of ^3H NA from the circulation. The drugs used in this study are known to pass through the placenta (Idänpää-Hiekkilä, Jouppila, Puolakka and Vorne

1971 Kivalo and Saarikoski 1970, 1972 Mirkkin 1973), but it is difficult to estimate what influence the use of these drugs may have had on the results.

Passage of noradrenaline and its metabolites into the foetus

The placenta may be compared functionally to a lipoprotein membrane. Fat-soluble substances pass through it more efficiently than water soluble ones, and non ionized more efficiently than ionized substances. Substances with a molecular weight less than 600 pass it easily into the foetus, while for molecular weights in excess of 1000 the placenta acts as a barrier (for ref., see Ginsburg 1971 Levy and Hayton 1973 Mirkkin 1973). The molecular weights of NA and its metabolites are low of the order of 200 but these compounds are hydrophilic. However on the basis of their low molecular weight it might be assumed that these compounds pass into the foetus with ease.

Passage of NA into the foetus The passage of ^3H NA added to the mother's circulation, into the foetus, observed in the present study was minimal and it can hardly be attributed any significance in the regulation of the foetal circulation as has been thought possible (Sandler *et al.* 1963 1964 Morgan *et al.* 1972). NA metabolites, on the other hand, reach the foetus easily and rapidly which shows that the placental enzyme barrier efficiently isolates the foetus from the effect of the transmitter substances of the mother's adrenergic nervous system. This observation must be interpreted as indicating that in the placenta *in vivo* rapid decamination and O-methylation take place, as was previously indicated by *in vitro* studies (Luschinsky and Singher 1948 Kivalo and Castrén 1967 Klinge 1968 Castrén and Saarikoski 1974).

No attempt was made at estimating placental NA inactivating capacity in the present study indeed, it is obvious that the placenta has a high NA metabolizing capacity (Bekaert *et al.* 1966 Thierry *et al.* 1967). Only when the quantity of CA in the circulating blood under goes a marked increase, as in pheochromocytoma, it is conceivable that there is a risk of NA traversal in such quantities that it could have a physiological effect. Strong placental degeneration associated with pheochromocytoma (see Batt *et al.* 1974) or *e.g.* with toxæmia, may also possibly increase the passage of NA into the foetus especially during labour. In this connection, remarkably high plasma NA concentrations were

found in some such patients (Saarikoski, Jäätelä and Ikonen unpublished data 1972). The placental and umbilical blood vessels are not sensitive to the effect of NA (Klinge, Mattila Penttilä and Jukarainen 1966 Dawes 1973) in contrast with the circulatory shunts of the foetus (Ehinger *et al* 1968 Bortus *et al* 1969 Aronson *et al* 1970 Owman *et al* 1973). It seems to be obvious that high NA levels in the maternal blood can be harmful to the foetus mainly as a result of foetal hypoxia arising from NA induced uterine arterial vasoconstriction and uterine contractions.

NA in foetal tissues It has been shown that the uptake of ^3H NA into tissues after intravenous injection depends on two factors, i.e. the proportion of cardiac output reaching the tissue and the endogenous NA content (for ref., see Axelrod 1965 Iversen 1967). In human foetal organs the NA content roughly parallels that of adult organs of other species indicating the presence of a functioning adrenergic nerve supply and of NA binding capacity at an early stage of development (Greenberg and Lind 1961 Read and Burnstock 1970 Hervonen 1971).

With the foetus *in utero* the activity passing through the placenta consisted almost totally of metabolic products of ^3H NA, and therefore the quantity of ^3H NA in the foetal tissues was indeed very low not even measurable in all instances. The ^3H NA which passes into the foetus is taken up mainly by the liver since of its total activity after 5 min ca. 15 % consisted of conjugated ^3H NA. Uptake by the other foetal tissues plays a very minor role in the foetal inactivation *in utero* of ^3H NA administered to the mother.

If a more considerable amount of ^3H NA enters the foetus as occurred with the foetoplacental unit experimental model and when the foetus was treated outside the uterus with its umbilical cord clamped the adrenergic uptake mechanism is obviously of significance in the inactivation of ^3H NA, because the greater part of the radioactivity found in the tissues under these circumstances was ^3H NA. Tissue uptake of ^3H NA, however seems to be a less important mechanism of inactivation than hepatic metabolism. This could be seen clearly in the extrauterine experimental arrangement where the quantity of radioactivity in the liver alone was on the average equal to the aggregate content of all other tissues examined. But, it is felt that with this particular arrangement the involvement of the liver is potentiated by the administration of the ^3H NA into the umbilical vein.

Metabolites of NA in the foetal tissues The distribution of ^3H NA metabolites in the foetal tissues *in utero* was for the most part similar in all tissues studied in that in the placenta and umbilical venous blood, the predominant unconjugated metabolite was ^3H MHPG-VMA. The results presented indicate that the placental metabolism is the main foetal protecting system. NA metabolites appear to have a particular affinity for the liver: the concentration of ^3H MHPG-VMA in the liver after 5 min was equal to that in the umbilical vein and was clearly higher than that in other foetal tissues or in the placenta, and 30 min after the administration of ^3H NA to the mother the concentration of ^3H MHPG-VMA in the foetal liver was distinctly higher than that in the umbilical vein, placenta and foetal tissues. These results approximate those obtained *in vitro* during rat liver perfusion. The liver accumulates metabolites of A over a prolonged period of time from the perfusing medium but not A (Lightman and Hems 1973).

With the foetoplacental system, after the addition of ^3H NA to the foetal circulation 10 to 25 % of the activity was found as unconjugated metabolites in the foetal tissues. The metabolic transformation of the ^3H NA was mediated mainly by the action of COMT as is the case in adult tissues also (Hertung and Axelrod 1961). This can be seen from the fact that in the tissues the concentration of ^3H NMN was ca. twice that of ^3H DHPG-VMA, and the concentration of ^3H MHPG-VMA equal or greater than that of the latter.

Conjugation of NA and its metabolites in the foetus Under different experimental arrangements ^3H NA, ^3H NMN and ^3H MHPG-VMA were found in conjugated form. It is obvious that ^3H NA and its metabolites can be conjugated in the foetus, because the proportion of activity in the foetal liver in conjugated form was significantly higher than in the umbilical venous, umbilical arterial and maternal peripheral blood and since the amount of conjugated activity in the umbilical venous blood during the first five min after ^3H NA administration to the mother was minimal. However the possibility cannot be completely excluded that ^3H NA and its metabolites conjugated in the mother might be transported into the foetus to some extent.

The ability of the foetus to conjugate ^3H NA and its metabolites with sulphuric acid was confirmed with the extrauterine experimental arrangement. Under these conditions with the umbilical cord clamped and the connection with the mother eliminated it was possible to measure the foetus own metabolic and NA-inactivating capacity under anaesthetic

SUMMARY

The material consisted of 44 fetuses (11–24 weeks) weighing between 15 and 420 g. The fetuses were obtained at the termination of pregnancy performed by a laparotomy approach.

A new method was employed for the determination of ^3H NA and its metabolites. The tissues were homogenized in 0.4 mol/l perchloric acid, neutralized to pH 5.4–5.8 and the metabolites separated by paper chromatography. Whatman no. 1 paper solvent systems: n-butanol/formic acid/water 70:12:15 v/v, 2 days old or n-butanol/formic acid/butyl formate/water 7:1:1:2 freshly prepared, running time 12 hours. The R_f values of NA and its principal metabolites were: NA, 0.18; NMN, 0.34; DHPG, 0.49; DHMA, 0.56; MHPG, 0.68; VMA, 0.75 and conjugated metabolites, 0.00–0.03. The quantity of radioactivity in each metabolite fraction was determined by combustion of the paper strip after staining in Kaartinen's sample oxidizer (Kaartinen 1969) to radioactive water with subsequent liquid scintillation counting.

The recovery of radioactive NA and its metabolites (mean \pm S.D.) was: NA, $94.1 \pm 3.4\%$; NMN, $97.4 \pm 2.0\%$; DHPG-DHMA, $96.6 \pm 3.0\%$; MHPG-VMA, $94.4 \pm 3.5\%$.

The study consisted of four parts involving different experimental arrangements.

1. The rate of passage of NA and of its metabolites through the placenta was studied by injecting 1 ^3H NA of high specific activity into the mother's circulation and determining the ^3H NA and metabolite content of the umbilical blood 1–5 min after the injection. It was found that ^3H NA disappears very rapidly from the maternal circulation; the half-life was estimated as ca. 30 sec. In the foetal umbilical blood radioactivity was detected 1 min after the injection and an equilibrium was established between the maternal and umbilical venous circulations within 2–3 min of the injection. The passage of ^3H NA into the foetus was minimal: only 10–12% of the radioactivity in the

umbilical blood was ^3H NA, the rest was metabolites. ^3H MHPG-VMA accounted for about 50 % of the activity within 2 min after the injection and subsequently ^3H NA is apparently efficiently metabolized to its end product in the placenta.

2. The distribution of NA and its metabolites in the foetal tissues their storage and further metabolic processing *in utero* were studied by injecting 1 ^3H NA into the mother's circulation and taking blood and tissue samples from the mother and foetus 5 30 and 240 min after the injection

When the foetus was *in utero* the activity traversing the placenta consisted almost totally of metabolic products of ^3H NA and for this reason the amount of ^3H NA in the foetal tissues was low. The ^3H NA which reaches the foetus was taken up primarily into the liver. The ^3H activity in the foetus is fairly uniformly distributed between the different tissues, the concentration is lowest in the brain and highest in the aortic bodies and the liver. The placental ^3H content was highest of all. Of the foetal tissues the liver had the highest ^3H content 5—30 min after the injection, but not after 240 min.

The pattern of ^3H NA metabolites in all the foetal tissues *in utero* was for the most part similar to that in the placenta and umbilical venous blood. ^3H MHPG-VMA was the predominant unconjugated metabolite in all the tissues. In the liver intestine, lungs and kidneys conjugated ^3H NA and ^3H NMN were detected 5 min after the ^3H NA administration to the mother and conjugated ^3H NMN and ^3H MHPG-VMA 30 min after the injection. The liver retained ^3H NA metabolites over a prolonged period of time.

3. The uptake, storage and metabolic capacity of the foetal tissues when challenged with a large quantity of NA were studied in the foetoplacental unit by injecting 0.1 ^3H NA or 1 ^3H NA, into the umbilical vein or jugular vein of the foetus. The umbilical cord was clamped 15 min after the injection and the ^3H NA and its metabolites in the foetal tissues were determined.

In the foetoplacental unit the quantity of radioactivity found in the brain per unit weight, was small, on the other hand the distribution of activity in the peripheral tissues was fairly uniform. In contrast with the situation in the foetus *in utero* in the foetoplacental unit the activity in the tissues consisted mainly of unmetabolized ^3H NA. It was present in greatest amounts in the heart, followed by the intestine, kidneys and adrenals. This result indicates that in these tissues active uptake serves as a mechanism of inactivation for circulating NA. In

the liver more than half of the activity was present in the form of conjugated metabolites. ^3H NA and ^3H NMN-sulphate. The quantity of tissue ^3H NMN was about twice that of ^3H DHPG-DHMA and ^3H MHPG-VIA. Thus it follows that in the foetus too O-methylation represents an important metabolic step for NA.

4 The ability of the foetus to conjugate ^3H NA and its metabolites with sulphuric acid was confirmed by injecting of 1 ^3H NA into the clamped umbilical cord. Fifteen min after injection ^3H NA and ^3H NMN-sulphate were detected in the foetal liver. In this anoxic condition the disappearance of ^3H NA from the foetal circulation was slower than in the adult.

Under normal conditions when the foetus is *in utero* the transfer of NA from the mother to the foetus is minimal and obviously has no significance in the regulation of the foetal circulation. The placenta efficiently takes care of the metabolic inactivation of any NA that may arrive from the mother both by deamination and O methylation. The foetus own NA inactivating capacity is also well developed. The most important tissue for such inactivation is the liver which is able to metabolize NA and its metabolites through MAO and COMT activity and by catalyzing sulphate conjugation. Conjugation is also believed to take place in the lungs, intestine, kidneys and adrenals.

The adrenergically innervated tissues of the human foetus have the capacity to actively take up NA already during the first half of intra uterine life, but as a mode of NA inactivation this pathway is thought to be less important than the hepatic metabolic inactivation.

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Seppo Saarikoski

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SUPPLEMENTUM 422

**CARDIORESPIRATORY RESPONSE
TO UNILATERAL
PULMONARY ARTERY OCCLUSION**

AN EXPERIMENTAL STUDY IN THE DOMESTIC FOWL

By
ESKO LÄNSIMIES

TURKU 1974

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SUPPLEMENTUM 422

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ABBREVIATIONS AND SYMBOLS

a	= arterial
b w	= body weight
CO ₂	= carbon dioxide
cm	= centimeter
d	= day days
ECG	= electrocardiogram
kg	= kilogram
KV	= kilovolt
min	= minute
ml	= milliliter
mmHg	= millimeters of mercury
mV	= millivolt
PRQ	= blood pressure/blood flow = mmHg/ml/sec
O ₂	= oxygen
P	= partial pressure
Q	= blood flow
sec	= second
	= venous
v _{O₂}	= oxygen uptake

2 INTRODUCTION

Homeothermic animals have a reserve of oxygen which will usually last for a few minutes. The rapid exchange of respiratory gases is possible because of the specialized pulmonary circulation is totally separated from the systemic circulation in full-grown mammals and birds. However the pulmonary circulations in these two groups of animals have developed from different origins. In mammals the separating interventricular septum of the heart is the primary septum. In the avian heart it is the secondary septum which is also encountered in some reptiles. The origin of aortic arches and pulmonary vessels also differ between mammals and birds (Foxon 1955).

Gas exchange in mammalian lungs occurs through a thin multilayer membrane. Gases move to and from the alveoli when blood flows through the alveolar capillaries. Thus a uniform pool model for respiratory gas transfer is formed (Piiper and Scheid 1972). Bronchial arteries supply lung tissues with oxygen-rich blood. This vascular system originates from the aorta or its branches and terminates on the right side of the heart. Most of the blood in these arteries however perfuses the capillary network that also receives blood from the pulmonary arteries (Daly and Hebb 1966). This blood flows to the left side of the heart through the pulmonary veins and is partly responsible for the so-called physiological shunt (Aviado 1965, Daly and Hebb 1966).

Avian species have air sacs that are absent in mammals. Avian lungs are fixed into the thorax wall and thus do not collapse when thoracotomy is performed (Burton, Beach and Smith 1968). Air flows through the trachea, primary bronchi (meso-bronchi) and secondary bronchi (dorso- and ventrobronchi, see Fig. 1). In the region of the tertiary bronchi (parabronchi) the air flow is unidirectional from caudal to cranial direction (Baselhoff 1951, Bouverot and Dejours 1971, Brackenbury 1972, Lasiewski 1972, Scheid, Slama and Piiper 1972). The tertiary bronchus, air capillaries and atria together form a hexagonal lung unit where the gas exchange occurs (Bræmer 1939, Akester 1971, King and Molony 1971, see Fig. 2). The gas exchange between

atria and air capillaries takes place by diffusion only (Hazelhoff 1951). The pulmonary arteries bring blood to this lung unit and it is then distributed to the vicinity of the air capillaries by blood capillaries. Thus a complex capillary network zone is formed (Fig 3). This network corresponds to the mammalian alveoli. Both counter current (Schmidt-Nielsen 1971) and cross current (Pilpar and Scheid 1972) principles for gas and blood flows have been proposed. Bronchial arteries originate from the aorta, carotid arteries or pleural arteries (Owen 1866, Königstein 1903, Westphal 1961). The blood flow through this vasculature as well as the exact organisation of the smallest pulmonary vessels is only poorly known (Akester 1971 b).

The body possesses anatomical, chemical and functional reserves in excess of its normal requirements (Kao 1972). At rest, acute reduction of the gas exchanging area must and can easily be tolerated. In mammals these maximal ventilation-perfusion fluctuations have been discussed by Nordenström (1954), Comroe (1965) and Aviado (1965). They confirm the very rapid normalisation of ventilation and circulation after acute pulmonary artery occlusion.

The capability of the avian ventilation and circulation to endure and compensate the acute reductions of the gas exchanging area is far less clearly understood and studied. In fact, there is no systematic study of cardiorespiratory responses to unilateral pulmonary artery occlusion in domestic fowl. The studies of Butler (1968) will be cited in detail.

In mammals chronic reduction of the gas exchanging area leads to a permanent reduction in the increase of systemic blood flow and in the formation of new pulmonary capillaries. The reduction of pulmonary blood flow may be 30 per cent without significant effects on the ventricle (Nordenström 1954).

The response of the avian lung to the chronic occlusion of one pulmonary artery has been studied by morphological methods only in our laboratory (Lämsä and Karkola 1973). Increases in enzymatic activities (phosphatases, esterases, amino-peptidases and reductases) suggesting vascular growth and tissue formation were observed. Several months after occlusion the enzyme activities describing special functions were somewhat different as compared to the unoccluded state.

3 REVIEW OF THE LITERATURE

The avian lung is apparently the most efficient gas exchange apparatus in air breathing vertebrates (Lasiewski and Calder 1971 Weibel 1973) although the effective ventilation/volume ratio is more unequally distributed than in mammals (Scheid and Piiper 1969) The blood flow through the avian lung shows a notably lower $\dot{Q}/\dot{V}O_2$ ratio than the other vertebrates (Johansen 1972)

3.1 Cardiorespiratory parameters of the domestic fowl

The respiration rate of the domestic fowl varies greatly depending on the recording situation Normal values from 20 to 40 breaths per minute have been reported (Piiper et al 1970 see also Table I)

The partial pressures of carbon dioxide and oxygen in the blood of the domestic fowl have previously been determined and are listed in Table I $PaCO_2$ values observed are rather constant 33-30 mmHg Venous PCO_2 is higher about 40 mmHg Lower values have been reported for capillary samples (Bouverot et al 1972) PaO_2 varies from 87 to 110 mmHg venous pressure for oxygen is lower about 40 mmHg Oxygen saturation is 98 per cent when arterial oxygen partial pressure is about 110 mmHg (Burton et al 1968) Arterial pH of unanesthetized domestic fowl is about 7.5 In anesthetized animals lower values have been reported In capillary blood samples the pH is higher

The blood pressure and heart rate of the domestic fowl have been reviewed by Akester (1971 a b) Table II summarizes the studies performed on unanesthetized animals All male and old female animals have a higher blood pressure and lower heart rate than young (4-6 months) female animals (Sturkie Weiss and Ringer 1953 Ringer Weiss and Sturkie 1955 Girard 1973) The role of gonadal hormones is however still obscure (Ringer et al 1957) Body temperature also has an effect on both heart rate and blood pressure (Rodbard and Tolpin 1947) Blood pressure and heart rate vary depending on age and sex The mean systemic arterial blood pressure ranges between 100 and 164 mmHg in young female animals the values reported are close to 140 mmHg Heart rates earlier reported vary within wide limits

from 284 to 378 per minute. Young female animals have a heart rate of about 350 per minute.

Table III summarizes the results of earlier studies of some hemodynamic parameters of the domestic fowl. The cardiac output was first determined by Sapirstein and Hartman (1959). Different methods produce values ranging from 143 ml/kg x min to 270 ml/kg x min. Stroke volumes are reported to be between 0.48 and 0.71 ml/kg depending on the age and sex of the animal and the method used. Systemic arterial peripheral resistance ranges between 20 and 31 peripheral resistance units (PRU).

Electrocardiology of the avian heart has been reviewed by Jones and Johansen (1972). An avian electrocardiogram was first recorded in 1913 by Buchanan (see Kisch 1951). The P-wave duration is 25 msec. PR-interval has been reported to be from 60-81 msec and QRS-duration about 30 msec (Zuckermann 1959, Kisch 1951).

3.2. Cardiorespiratory response to stress in the domestic fowl

The response to hypoxia in the domestic fowl is dependent on the degree and duration. Butler (1967) and Richards (1971) observed that the respiration rate of intact unanesthetized chickens increases during the initial stages of progressive hypoxia due to rebreathing of respiratory gases. During later stages of hypoxia the respiration frequency increases further. An intact vagus nerve is essential for an increase in respiration frequency. Richards and Sykes (1967) reported that in hens anesthetized with pentobarbitone the respiration rate decreased after an initial increase when progressive hypoxia had reached an arterial pressure of 50 mmHg. A similar kind of increase in the respiration rate and ventilation is observed during hypoxia produced by reduction of oxygen in the respiration gas (Ray and Fedde 1969, Bouverot and Leitner 1972). The heart rate increases in the initial phases of hypoxia and then decreases in animals that are awake. The initial tachycardia is probably dependent upon autonomic innervation but the final bradycardia is due to a direct anoxic effect in the cardiac tissue (Butler 1967). The same order of changes in cardiac rate is observed in pentobarbitone-

anesthetized animals too (Richards and Sykes 1967) An increase in the heart rate is found to occur only below the arterial PO_2 of 70 mmHg in anesthetized animals (Ray and Fedde 1969) The mean systemic arterial blood pressure during the early stages of hypoxia decreases only slightly (Richards and Sykes 1967) or not at all (Butler 1967) In the later stages of progressive hypoxia there is a marked decrease in blood pressure (Butler 1967 Richards and Sykes 1967 Ray and Fedde 1969) Cardiac output increases during hypoxia due to increases in both heart rate and stroke volume (Butler 1967 Jones and Johansen 1972) The domestic fowl is less tolerant to hypoxia than pigeon or duck (Butler 1970)

Studies concerning the cardiorespiratory response to hypercapnia in the domestic fowl have given controversial results The respiration rate has been found either to increase (Leitner 1972) or to decrease (Richards and Sykes 1967 Ray and Fedde 1969) in unanesthetized hypercapnic animals Carbon dioxide sensitive receptors are supposed to be in the lungs of the domestic fowl (Peterson and Fedde 1968 Eaton Fedde and Burger 1971) These receptors are located in the arterial system (Bouverot and Leitner 1972) In anesthetized animals hypercapnia induces a decrease or an increase in respiration rate but an increase in the amplitude of respiration increases the total ventilation This response has been demonstrated in anesthetized tracheal cannulated animals and mechano receptors have been proposed (Bouverot and Leitner 1972) although the presence of pulmonary stretch receptors has been denied by Eaton et al (1971) The ventilatory response to hypercapnia is mediated mainly through the vagus nerve and only partly through the sympathetic nervous system (Burger 1968 Akester and Mann 1969 Cook and King 1969 Fedde 1970) Vagal influence contracting the smooth muscles of the tertiary bronchi controls the ventilation and prevents a CO_2 deficit during hyperventilation (King and Cowie 1969) Heart rate and blood pressure are only slightly modified by moderate hypercapnia (Richards and Sykes 1967) In unanesthetized animals bradycardia and a decrease in cardiac output have been reported (Butler 1967 a b) Ray and Fedde (1969) observed an increase in blood pressure and heart rate during normoxic hypercapnia

in anesthetized animals

Asphyxia produces effects similar to or slightly greater than those observed during hypoxia alone. The heart rate will fluctuate but bradycardia occurs at the end of the asphyxic period (Richards and Sykes 1967)

Cardiorespiratory response to the occlusion of the left pulmonary artery in the domestic fowl has been studied in very few and acute situations only. The respiration rate was rather high 49 per minute and heart rate rather low 293 per minute in young anesthetized animals with open thorax and occluded left pulmonary artery of unknown period. Further no control measurements were made (Rodbard et al 1949). From the results of Peterson and Fedde (1968) and Henry and Fedde (1970) it can be concluded that the respiration rate is increased after unilateral occlusion of both the pulmonary artery and the vein but the sensitivity of the respiration to CO_2 is independent of the blood flow through the lung. In these studies the animals were anesthetized and their thoracic and abdominal cavities were opened and they were ventilated by a respirator. A slight increase of systemic arterial blood pressure was also observed in their studies. Burger (1968) observed that when the content of CO_2 in the ventilation gas was increased from zero to 15 per cent respiration amplitude increased more in animals with an occluded left pulmonary artery than in animals with intact pulmonary circulation. The difference was however slight. Burton et al (1968) reported a pulmonary arterial hypertension after unilateral occlusion of the pulmonary artery.

Enzyme histochemical studies after occlusion of the left pulmonary artery have been performed in our laboratory (Lämsmäe and Karkola 1973). Immediately after the occlusion non-specific esterase activity increased in the epithelial cells and the tertiary bronchi. Naphthylamidase reaction increased intensely in the epithelium and adjacent structures after the occlusion. Capillary network zone showed pronounced tetrazolium reductase activity in a corona like circle around bronchi. It was concluded that the active transport mechanisms seem to be disturbed immediately after the occlusion but no

severe ischaemic changes were observed. A temporary activation of energy yielding process was noted in the capillary network near the tertiary bronchi. A proliferative or inflammatory process was perceptible in the epithelium of the tertiary bronchi and the atrial walls.

The diving pattern is a universal response to hypoxia produced for example by submerging into water. This is a phylogenetically old reflex and all vertebrates seem to respond in a similar manner. In natural divers these oxygen saving adjustments operate at a very high degree of precision (Andersen 1966). The avian response to diving has been reviewed by Jones and Johansen (1972). In diving animals apnea is promptly followed by bradycardia, a redistribution of the regional blood flow and a decrease in cardiac output. These changes serve to economize the available oxygen stores (Folkow, Nilsson and Yonce 1967; Jones and Johansen 1972). A simulated diving bradycardia in ducks is more dependent upon the progressive hypercapnia than on the increasing anoxia (Feigl and Folkow 1963). Mean arterial blood pressure has been reported to decrease only slightly (Folkow *et al.* 1967; Butler and Jones 1971) although the animals showed a generalized vascular constriction response. Myocardial contractility may also be decreased (Folkow *et al.* 1967) although the myocardial circulation is proportionally increased (Johansen 1964). The nervous control of these responses is mainly vagal but evidence of inhibitory sympathetic tone has also been proposed by Folkow *et al.* (1967). An increase in the resistance of the pulmonary outflow and the reduction of the pulmonary artery caliber may be caused by the direct effect of the low alveolar PO_2 or by chemoreceptor reflex (Eliassen 1960; Aakhus and Johansen 1964; Jones and Johansen 1972). The immediate post-dive period results in a rapid normalisation of the observed changes. Sometimes this correction is over-shooting and usually of short duration (Folkow *et al.* 1967; Jones and Johansen 1972).

3.3 Morphology of the avian lung

The cross section of a tertiary lung unit varies from 300 to 500 μm the atria from 100 to 200 μm (King and Molony 1971). The blood capillaries are about 7 μm and the air capillaries about 10 μm in diameter. The air-blood barrier thickness varies from 0.1 to 0.7 μm and the surface lining of the airways is 0.015 μm in thickness. The surface lining is constructed of three layers of 0.005 μm in thickness. Two of these layers are osmiophilic and the one between them is non-staining (Patrik and Riedel 1968 a). The diameter of the osmiophilic inclusion bodies varies from 0.5 to 1.0 μm (Jones and Radnor 1972).

Morphology of the gas-exchanging part of the avian lung is schematised in Figures 2 and 3. The small arteries and veins run parallel with the tertiary bronchi. The smaller vascular loops carry blood from these small arteries to the veins. Air capillary endothelium gradually decreases and becomes indistinct and terminally fuses with the epithelium of the blood capillaries. According to Duncker (1972) the blood capillaries originate from septal arterioles (Fig 2) and lead towards the lumen of the tertiary bronchus. By the lumen the capillaries converge to form venules and emerge from the tertiary bronchi as small veins. Hence it follows that the length of the capillary involved in the gas exchange corresponds approximately to the thickness of one tertiary bronchial lung unit. These observations confirm the cross-current exchange principle as described by Scheid and Piiper (1970). In avian lungs the tertiary bronchi are surrounded by a sheath of air capillary/blood capillary meshwork in which gas exchange takes place. In this model blood vessels have gas exchange contact with tertiary bronchial gas inside a single cross-sectional element of the tertiary bronchus only. Thus blood flows effectively at a right angle to the air tube axis. The arterial blood originates from a mixture of affluent blood whose partial pressure values vary along the length of the air tube (Scheid and Piiper 1970).

Air and blood are separated by a continuous cellular lining (Breuer 1939, Bargmann and Knoop 1961, Nagaishi et al 1964). In electron microscopical studies the osmiophilic membranes have been seen on the epithelial surfaces of the tertiary

bronchi and the atria (Tyler and Pangborn 1964) and air capillaries (Patrik and Riedel 1968 a b) These membranes are probably responsible for the surface tension-reducing properties of the avian lung extracts (Tyler and Pangborn 1964) Osmiophilic lamellar inclusion bodies have been found intraepithelially near the site of the surface-active substance production (Adamson and Bowden 1973) as well as in the epithelium of air sacs (Carlson and Beggs 1973) These bodies are seen already during the fetal period (Jones and Radnor 1972 a b) The osmiophilic bodies are probably the side products of surfactant synthesis rather than the immediate source of this substance (Adamson and Bowden 1973) Studies of the innervation of the avian lungs reveal that vagal innervation is dominant (King and Cowie 1969 Akester and Mann 1969 Cook and King 1969 McLelland / Cook and King 1972)

4 PURPOSE OF THE PRESENT STUDY

In order to study the adaptativity and efficiency of the circulation and gas exchange in the domestic fowl, unilateral pulmonary artery occlusion was performed and the immediate and long term responses were studied by functional and morphological methods:

- 1 How does the exchange of respiratory gases change after occlusion?
- 2 What are the hemodynamic responses to occlusion?
- 3 How does the electrical activity of the heart respond to the changes in circulation after occlusion?
- 4 How does the blood- air barrier and bronchial vasculature change after occlusion?

5 MATERIAL AND METHODS

A total of 101 white Leghorn chickens were used. The animals used in hemodynamic and respiratory studies were young (3-4 months) healthy females. In morphological studies the ages at the time of operation and registrations varied more than in cardiorespiratory experiments. A full list of animals used in different experiments is given in Tables IV-VI as an appendix to this report. The animals used in cardiorespiratory measurements were used in morphological studies too.

Water and commercial food were provided ad libitum. The cages were indoors, 150 x 150 x 90 cm in size. Four to six animals were in each cage. The temperature was kept constant at 17-18°C. The bedding material was raw wood shavings. Diurnal rhythm was kept constant by artificial illumination from 7 am to 6 pm.

5.1 Operative procedures

An assistant kept the feet and head of the animal gently against the table during operation and registrations. The eyes were covered and the animals were lying on their right side. The animals were calm during operative procedures. Polyethylene tubes 1 mm wide were introduced under local anesthesia of lidocaine through the brachial artery and vein into the aorta and anterior caval vein to the level of the right atrium for pressure recordings, blood sampling, and cardiac output determinations. The same tubes and instrumentation were used on all animals. The operative method to produce the unilateral pulmonary artery occlusion was modified from Rodbard, Brown and Katz (1949) and Burton, Besh and Smith (1968). A blunt dissection was made through the left side of the pectoral muscle using local anesthetic. The intercostal space was further anesthetized from the angle of the ribs towards the sternum. The intercostal space was opened and the ribs parted. The left pulmonary artery was easily recognized within a sheath of connective tissue. The pulmonary artery was dissected free from the surrounding tissue by a specially designed curved wire hook (10 cm long and the radius of the hook was 4 mm). The artery was pulled laterally (upwards) and closed by surgical

silver clips. The intercostal and muscle incisions were closed with silk stay sutures. The animals were kept in separate cages for one day postoperatively and then replaced to their original cages. The instruments used in the operation were clean but not sterilized.

The effect of position on respiration was studied by turning the animal on each side because mammalian respiration is dependent of position (Comroe 1965 Kao 1972)

5.2 Cardiorespiratory recordings

Respiration measurements were performed on 25 young female animals (Table IV). Five of these were controls, nine were sham-operated controls and eleven were occlusion-operated. Five of the occlusion group were followed for one week and six for two weeks. Registrations were carried out before operation, three minutes, 15 minutes and 30 minutes after occlusion and one or two weeks after operation. The thorax was closed between registrations of three and 15 minutes. The respiration rate was measured visually during a 30 sec period. Arterial and venous blood samples were simultaneously drawn into heparinized glass syringes anaerobically for immediate pH, PCO_2 and PO_2 determinations. These were performed on IL-227 equipment (Instrumentation Laboratory, Milan, Italy) at $37^{\circ}C$ and corrected for $41^{\circ}C$ (Piiper *et al* 1970) according to the nomogram of Kelman and Nunn (1966). Blood oxygen saturations were measured from the same samples by an oxymeter (Kipp, Delft, Netherlands).

Hemodynamic measurements were performed on 39 female animals (Table V) with similar lining as in respiration measurements. Ten of the animals served as controls, nine were sham-operated controls and 20 were occlusion operated. 20 of these five were killed 15 minutes after operation, nine were controlled for one week and six for two weeks after occlusion of the left pulmonary artery.

Systemic arterial pressure (mmHg) was registered through a cannula in the aorta. Elema pressure transducers calibrated to the level of the heart and a Mingograph 34 recorder (Elema, Sweden) were used. The mean pressure was integrated by the

recorder. The heart rate was measured from simultaneous ECG recordings or from pressure recordings from periods of 20-30 sec. Cardiac output and stroke volume were determined by the indicator dilution method as described by Sturkie and Vogel (1959) and modified by Polkow et al (1967). Indocyanine green (Cardiogreen^R Hynson and Westcott USA) was injected in small boluses into the anterior caval vein near the right atrium. Arterial blood samples were drawn with a pump (Perfusor Braun GFR) through the cannula from the aorta. The dye concentrations were determined with Cardiognost apparatus (Atlas Werke GFR). Three to four determinations were performed during each recording period. Cardiac output was expressed in ml/min kg b w and stroke volume in ml/kg b w. The mean peripheral systemic arterial vascular resistance was calculated by dividing the mean systemic arterial pressure value (mmHg) by the total cardiac output value (ml/sec). This ratio is used as a unit for peripheral vascular resistance (PRU).

Electrocardiographic registrations were performed in 23 occlusion-operated female animals (Table VI). The animals were studied at regular intervals up to six months. Needle electrodes were fixed subcutaneously to points 2 cm laterally from the rostral edge of the keel to the right and left sides. The third electrode was inserted into the area of the caudal end of the keel. These points were considered to correspond the human limb leads and were optimal because if the electrodes were applied under the tight skin of the limbs the animals became restless. It is difficult to keep the exact positions of the wings and legs during the long follow-up period. In subsequent recordings the localisations were confirmed by puncture scars. Registrations were made by Mingograph 34 using bipolar recordings on three channels simultaneously. The mean QRS axis was determined from two recordings.

§ 3 Statistical methods

The method of statistical analysis was the Student's T-test. When a comparison is made between two means the p-value is given as a measure for the significance.

5.4 Morphological methods

In morphological studies 80 animals were used. Immediately after killing following various time intervals from the sham- or occlusion operation the sternum with the keel was removed and occlusion was ascertained as well as the exact location of the tubings. Then both lungs were visually examined. Tissue samples of 0.5 cubic centimeters were taken from the ventral edge of both lungs. Then the lungs were gently removed from the thorax and macroscopically examined. Similar tissue samples were taken from dorsal parts of the lungs. These are sites where the tertiary bronchi are most abundant (Duncker 1972). All samples were immediately fixed with neutral or acetic acid-formalin-alcohol (Lillie) (Vartio et al 1966). Serial paraffin sections were stained either with hematoxylin-eosin using Verhoeff's or Weigert-Domagk's stain. A total of more than 1,000 tissue slides were screened microscopically and demonstrative frames were photographed. One control and one long-term occlusion-operated animal were selected for ultra-microscopical analysis. Sixteen tissue samples were immediately fixed with glutaraldehyde solution for six hours, then post-fixed with osmium tetroxide for two hours and embedded in epon to be sectioned with Porter-Bloom microtome. After sectioning the samples were stained with lead citrate for transmission electron microscopy, the with toluidine blue for light microscopical analysis. With this technique the osmophilic structures survived. The sections were examined in a Siemens Elmiskop I A with an accelerating voltage of 60 kV.

In order to visualize bronchial vasculature microangiographical studies were made according to the method described by Wendelin and Lindgren (1970). Right hems were used. Three animals were studied 15 minutes after occlusion. Five animals including the sham-operated control were studied one week after the operation. The animals were heparinized before killing. The thorax was immediately opened and the ascending aorta was cannulated just above the pericardium. The aorta was then closed at the height of the liver. Both ventricles were opened to allow the free flow of the angiographic agent. One per cent radiopaque yellow suspension was used as the injection

recorder The heart rate was measured from simultaneous ECG recordings or from pressure recordings from periods of 20-30 sec Cardiac output and stroke volume were determined by the indicator dilution method as described by Sturkie and Vogel (1959) and modified by Folkow et al (1967) Indocyanine green (Cardiogreen^R Hynson and Westcott USA) was injected in small boluses into the anterior caval vein near the right atrium Arterial blood samples were drawn with a pump (Perfusor Braun GFR) through the cannula from the aorta The dye concentrations were determined with Cardiognost apparatus (Atlas Werke GFR) Three to four determinations were performed during each recording period Cardiac output was expressed in ml/min kg b w and stroke volume in ml/kg b w The mean peripheral systemic arterial vascular resistance was calculated by dividing the mean systemic arterial pressure value (mmHg) by the total cardiac output value (ml/sec) This ratio is used as a unit for peripheral vascular resistance (PRU)

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5.3 Statistical methods

The method of statistical analysis was the Student's T-test When a comparison is made between two means the p-value is given as a measure for the significance

6.1 Cardiopulmonary response to occlusion of the left pulmonary artery

The results of the respiration studies are given in figures 4 and 5. The respiration rate (Fig 5) of the animals preoperatively was 41 ± 2 breaths per minute in the occlusion-operated group and 40 ± 4 breaths per minute in the sham-operation group. The respiration rate increased to 74 ± 5 per minute three minutes after occlusion. This increase was significant ($p < 0.001$). The significantly higher respiration rate (59 ± 5 , $p < 0.01$) persisted up to 30 minutes after the occlusion operation and had levelled off to control value during the first postoperation week. The sham operation did not alter the respiration rate significantly ($p > 0.10$) during the 30 minutes after the manipulation of the pulmonary artery.

The arterial PO_2 (Fig 4) in the preoperative situation was 107 ± 2 mmHg and the respective saturation 91 ± 1 %. In the sham operation group the respective values were 98 ± 1 mmHg and 97 %. Three minutes after occlusion the arterial PO_2 decreased significantly ($p < 0.001$). The mean value was 87 ± 4 mmHg and the respective saturation 72 ± 5 %. Cyanosis was distinctly visible at this stage in occlusion-operated animals but not in the sham-operated control animals. After 15 minutes this hypoxia had vanished and the PaO_2 had returned to 100 ± 1 mmHg. This increase from a value of 3 minutes to a value of 15 minutes was significant ($p < 0.01$). Oxygen saturation remained significantly lower ($p < 0.001$) for 15 minutes after occlusion. Venous oxygen tension remained unchanged after occlusion and thus the arterio venous oxygen difference decreased. The sham operation did not alter significantly the arterial oxygen tension nor saturation ($p > 0.10$) for 30 minutes after the operation. The slight decrease in the PO_2 was significant ($p < 0.05$) in contrast to the occlusion-operated group where no change could be observed.

Arterial PCO_2 (Fig 5) was 28 ± 1 mmHg in the occlusion-operated group and 22 ± 1 mmHg in the sham-operated controls. Occlusion of the left pulmonary artery produced a mild hyper-

capnia PaCO_2 was 38 ± 2 mmHg at three minutes and 34 ± 1 mmHg at 30 minutes after occlusion. Both these values were significantly above the mean value before operation ($p < 0.001$). Arterial hypercapnia levelled off within two weeks following occlusion. In the sham-operated control animals PaCO_2 showed a slight significant decrease during the 30 minutes after the operation ($p < 0.05$). The venous PCO_2 showed a clear increase in occlusion-operated animals.

In the control situations before the operation the pH (Fig 5) of arterial and venous blood was 7.51 ± 0.01 and 7.47 ± 0.01 respectively. A decrease to 7.42 ± 0.01 and 7.41 ± 0.01 respectively was observed three minutes after occlusion. These decreases were statistically significant ($p < 0.001$) and remained for 30 minutes but vanished within the first post-operative week. In the sham-operated control group the mean pH values were higher than in the occlusion group but did not change significantly ($p > 0.10$) after the operation.

Turning of the animals on each side for 15 minutes did not change arterial nor venous PO_2 or PCO_2 nor pH. The systemic arterial blood pressure as well as heart rate remained essentially unchanged during these procedures.

The results of the hemodynamic determinations are given in Figures 6 and 7. Before operation the systemic mean arterial blood pressure (Fig 6) was 152 ± 5 mmHg. No significant ($p > 0.10$) changes were observed throughout the observation period although a slight trend to increasing blood pressure during 30 minutes and then a decrease to even below the initial control values was observed. The sham-operated control animals had essentially unchanged pressure recordings (160 ± 6 mmHg, $p > 0.10$) during the 30 minutes observation period after the operation.

Systemic arterial peripheral vascular resistance (Fig 7) was 11 ± 2 PRU in control situation and decreased to 9.5 fifteen minutes and 6.5 PRU seven days after the occlusion operation. The change from the preoperative level to the one week value was almost significant ($p < 0.10$). Stroke volume (Fig 7) was 1.7 ± 0.2 ml/beat per kg body weight before the

operation Fifteen minutes after occlusion the mean stroke volume remained unchanged One week after occlusion the stroke volume had increased up to 2.6 ± 0.4 ml/beat/kg b w ($p < 0.025$)

Cardiac output was 604 ± 77 ml/min/kg b w in 13 animals before operation In the animals which were used for the occlusion operation the mean cardiac output value before surgical procedures was 661 ± 102 ml/min/kg b w The mean cardiac output 15 minutes after occlusion was essentially the same as before operation One week after occlusion the cardiac output had increased to a mean value of 960 ± 200 ml/min/kg b w ($p < 0.10$)

The heart rate of the animals in this study was different in the two experimental groups one for hemodynamic studies and the other for electrocardiographical studies In the hemodynamic group the control value was 317 ± 10 per minute and for sham-operated animals 361 ± 15 per minute The 23 animals of the ECG group had a mean heart rate value of 362 ± 8 per minute before operation The response to the operation was however, similar in both groups The occlusion-operated animals had a progressive bradycardia This bradycardiac response lasted for up to 30 minutes and was significant in both groups ($p = 0.02$ and $p < 0.001$) The bradycardia normalized within one day, but could be observed in the hemodynamic group even two weeks after occlusion but this late fluctuation in the heart rate was not significant ($p > 0.10$) The sham-operated animals of the hemodynamic group showed no variation ($p > 0.10$) for 30 minutes after the operation

The results of the electrocardiographical registrations are given in Fig 8 and Table VII The P-wave duration was 19 ± 0.5 msec before operation This changed immediately but only slightly (21 ± 0.7 p < 0.01) Later after the occlusion operation the P-wave normalized the P-wave amplitude was 0.047 ± 0.007 mV before the operation It increased during the first post-operative day to the value of 0.108 ± 0.006 mV ($p < 0.001$) The decrease in the P-wave amplitude was statistically significant ($p < 0.01$) from the third post-operative day (0.094 ± 0.006 mV) to the two weeks value (0.065 ± 0.003 mV)

The PR-interval was 67 ± 1.6 msec before operation. There was a slight increase to 80 ± 2.2 msec immediately after the occlusion operation ($p < 0.001$) but the interval shortened to the preoperative level during the first day.

The QRS-interval was 21 ± 0.8 msec before the operation. After occlusion the interval increased up to 23 ± 0.9 msec ($p < 0.05$) and remained at a higher level for two weeks after occlusion. The mean frontal plane electrical QRS-axis pointed to $-67 \pm 6^\circ$ before the occlusion operation, indicating a clear left ventricle preponderance. The occlusion caused a distinct leftward shift ($54 \pm 8^\circ$) of the axis direction but this was not significant and the QRS-axis had returned to the preoperative direction during the second postoperative week. Although they are not indicated in Fig 8, the registrations were performed in 16 animals after three weeks. The P-wave duration was 19 msec, PR-interval 73 msec, QRS-duration 22 msec, P-wave amplitude 0.065 mV and the mean electrical axis pointed to -68° . In the registration performed during several months of fewer animals, no changes could be observed as compared to the 2-3 weeks situation after the occlusion operation.

6.2 Morphological response to occlusion

Morphological findings in control animals and in the right lungs of the occlusion-operated ones showed that no changes occurred during the observation period. The tertiary bronchioli were 200-300 μm in diameter, the atria were 100 to 200 μm in diameter. The capillary network zone was 130 to 300 μm in thickness with air capillaries approximately 15 to 20 μm in diameter. The blood capillaries never exceeded 20 μm , usually the diameter varied between 7 and 10 μm (Fig 9).

Microangiographical studies of both lungs of the control animals and the right lungs of the occlusion-operated animals revealed that the injection of radiopaque substance into the aorta gave only a slight contrast to the capillary network zone. The atrial septa were also visualized (Fig 10) and the injected substance came away from the lungs through the pulmonary veins.

The tissue between air and blood was very thin 0.3 - 0.6 μ m judged from the electron micrographs (Fig 11). The basal membrane between the endothelial cells was 0.12 - 0.15 μ m in thickness. Small vesicles could be detected on either sides of the basal membrane. The air capillaries were regularly covered by a thin osmiophilic lining (Fig 12). The thickness of the lining was about 0.01 μ m and it was formed from two osmiophilic membranes with a narrow space between them. Osmiophilic inclusion bodies were seen sometimes they were stretched.

Immediately after occlusion of the left pulmonary artery the lung resembled pulmonary oedema with enlarged interlobular and interatrial septa and with an increased amount of blood cells in the capillary network zone. From the first week onwards the lung was macroscopically firmer as indicated by palpations. A profuse arterial blood flowed from the cut of tissue sampling. Microangiographic substance stained the occlusion-operated lung bright yellow as compared to the hardly visible colorization of the right lungs of the occlusion-operated animals and both lungs of the control animals. In microangiographs (Fig 13 and 14) of the occlusion-operated lungs the pulmonary venules and the capillary network zone were more distinctly radiopaque by the infusate from the aorta through the bronchial arteries as compared to the control lungs. This was seen in the animals already one week after occlusion.

After occlusion the thickness of the interlobular septum increased and it contained more connective tissue than the control lungs (Fig 15). The tertiary airways and atria were small and irregular. The interatrial muscles were unchanged. After occlusion the capillary network zone gradually lost its regular structure and small vessels with greater lumen than in the capillaries were now observed. In contrast to the close vicinity of the air and blood in normal lungs these new vessels were distinctly apart from the air capillaries and located in the middle of the capillary network zone. The air capillaries were enlarged and the blood capillaries were reduced in number (Fig 16). From four to six months after the operation the occluded lung resembled the mammalian emphysematous lung. The interlobular septum was thick and collagenous. The interatrial

septum muscles were greatly reduced. Air capillaries were large up to $110\mu\text{m}$ in diameter. The new blood vessels found in the capillary network zone were up to $70\mu\text{m}$ in diameter and distinctly apart from air capillaries.

Electron micrographs (Fig 17 and 18) of the lung with four months of the post-occlusion period showed that the thickness of the wall between the capillary and blood vessels was 1 to $2\mu\text{m}$ and thus many times thicker than in the normal lungs. The basal membranes were irregular and had become thick. The osmiophilic lining of the air spaces was seen however. The intraepithelial osmiophilic inclusion bodies ($0.2 - 0.6\mu\text{m}$ in diameter) could still be found but they were definitely fewer.

7 DISCUSSION

In mammalian lungs the response to unilateral pulmonary artery occlusion is a marked increase in bronchial circulation (Weibel 1960). The increased bronchial circulation to the lung takes part in respiration gas exchange (Bloomer et al 1949). In dogs biochemical effects are quantitative changes in pulmonary phospholipids associated with the production of surfactant but if the post-occlusion period is long no differences were observed by Harlan Margraf and Said (1966). In this laboratory studies of the adaptation to pulmonary artery occlusion with special reference to pulmonary to systemic arterial anastomoses and respiratory distress syndrome have been performed (Thomson Linsimies and Linsimies 1972) and this study is a part of it.

The effects of the operative procedures as such on the registered parameters can be considered minimal as shown in the sham-operated animals. Thus this species of animal is suitable for this kind of study where rather massive surgical intervention upon the organism is made. The anatomical feature of adhesive pleurae and the easy handling of the domestic fowl further encouraged the use of this avian species in the study of lung physiology. The results obtained cannot directly be applied in the mammalian physiology but the repetition of evolution during foetal life makes the thorough understanding of comparative physiological aspects of respiration important.

In this study the respiration rate observed in the control situation was slightly higher than in previous studies (Table I). Burton et al (1968) used older animals and Piiper et al (1970) used animals with tracheal cannulation. The pronounced tachypnoea of 74 breaths per minute in the occlusion operated animals was normalized during the first post-operative week. Whether this tachypnoea increased the actual ventilation was not determined in this study but it is probable according to the studies of Peterson and Fedde (1968) and Bouverot and Leitner (1972). The lack of changes in the sham-operated animals is a further indirect evidence for the physiological significance of the increase in the respiration rate.

In the control situation PO_2 and PCO_2 were within the limits given by previous authors (Table I) Occlusion was followed by a short hypoxic period and a visible cyanosis which was not found in the sham-operated animals The hypoxia lasted only a short period as compared to the tachypnoeic period The arteriovenous oxygen difference decreased after occlusion but was unchanged or rather increased after the sham-operation Hypocapnia hyperpnoea and pH decrease appeared immediately after occlusion and persisted longer than hypoxia In sham-operated animals these changes were absent or even opposite to those observed in the occlusion operated animals

Clear hemodynamic responses to occlusion were observed The heart rate is under very close autonomic control and tends to vary in different experimental conditions This may explain the large variation in earlier reports (Table II) and the difference observed in this study between the two experimental groups and sham-operated group before operation (Fig 6) The response to occlusion and the absence of change in the sham-operated group however indicates the effect of occlusion The animals with a higher initial heart rate showed a greater decrease in heart rate than the other group with a lower initial heart rate According to Steinschneider and Richmond (1970) the law of initial value explains many of the differences observed in the heart rate responses of for example new born mammals The results of Rodhard et al (1949) obtained in anesthetized animals are in accordance with the results obtained in anesthetized animals are in accordance with the results obtained in this study bradycardia was observed after the occlusion The mean systemic arterial pressure was of the same magnitude as in this study (fig 6) as reported by previous authors (Table II) Significant changes were not observed after occlusion nor after sham-operations

Due to different methods and ages of the animals the cardiac output values registered in the control situation in this study (Fig 7) are considerably higher than those observed in earlier studies (Table III) In ducks using the same method as in the present study comparable values of 250-560 ml/min/kg b w have been reported (Sturkie 1966 Folkow et al 1967

Jones and Holston 1970) Allometric analysis of resting birds gave a value of $840 \text{ ml/min/kg b w}$ for a bird of the size used in the present study (Lasiewski and Calder 1971) The stroke volume value obtained in this study in the control situation ($1.7 \text{ ml/beat/kg b w}$) was greater than the values given by previous authors (Table III) but very close to the values ($1.37 - 2.45 \text{ ml/beat/kg b w}$) of Sturkie (1966) and Folkow *et al* (1967) obtained in ducks by the same method as used in this study The occlusion had no immediate effects on cardiac output stroke volume nor systemic arterial peripheral vascular resistance One week after occlusion these parameters had changed uniformly

Acute hypoxia and hypercapnia are both typical features of the diving pattern Domestic fowl is more sensitive to hypoxia than strong flyers and divers (Butler 1970) Thus the response to acute hypoxic and hypercapnic stress may come later and be less accurate than in the natural divers (Andersen 1966) In the presence of ventilation the simultaneous hypoxia and hypercapnia with hyperpnea and later bradycardia clearly indicate an increased vagal tone The vagus nerve is found to be the main regulator of the diving pattern (Jones and Johansen 1972) The receptors in the avian lungs are chemo- and mechanoreceptors (Eaton *et al* 1970 Bouverot and Leitner 1972) which both may be involved in the diving response These receptors are mainly in connection with vagal (Akester and Mann 1969) but also with sympathetic nervous systems (Barnett and Malmfors 1970 Fedde 1970) The rapid change in the ventilation perfusion ratio probably through nervous reflexory mechanisms affects the bronchial muscle tone and tends to normalize the ventilation perfusion ratio This mechanism has been found in hypoxic birds by King and Cole (1969) Scheid and Piiper (1970) and Lasiewski (1971) According to Scheid and Piiper (1970) in avian lungs it is possible to have a higher end tidal PCO_2 than in arterial blood In the sham-operated animals on contrary to occlusion operated ones there was a slight decrease in the arterial PCO_2 This may indicate a stress like action of the sympathetic nervous system The adaptative changes in circulation and the changes in the tone of the autonomic nervous system makes the use of available ventilation and perfusion more effective The determination of the exact role of the vagus nerve will require

direct recordings of the nerve impulses and the blocking of the autonomic receptors in connection with the occlusion

The increases in mean cardiac output and stroke volume values as well as the decrease in peripheral arterial vascular resistance may be considered as compensatory reaction to the reduction of respiratory tissue. The decrease in arteriovenous oxygen difference may indicate an increased circulation velocity with a shorter time for oxygen intake and consumption. A similar dynamic response as shown in this study during and after loading of the circulation has been observed in other avian species. Jones and Holeton (1972) observed increases in cardiac output, stroke volume and heart rate in ducks during simulated high altitude hypoxia. Feigl et al (1963) and Polkow et al (1967) observed an enormous increase in cardiac output and other parameters during the post dive period in ducks.

The effect of occlusion on the electrocardiographic parameters was similar to the changes found in pulmonary embolism. The increase in the P-wave amplitude usually called P-pulmonale is a sign of the right atrial overload. A temporary and slight slowing down of the conduction velocity, PR- and QRS-intervals confirmed this enlargement of the heart. A clearcut deviation of the frontal plane QRS-axis to the left also was suggestive of the analogy to pulmonary embolism. In man and dog occlusion of the pulmonary artery is most often followed by a left-ward shift of the QRS-axis (Zuckermann et al 1950, Lynch, Stein and Bruce 1972). In the present study the shrinkage and atrophy of the left lung (possibly resulting in a better electrical conductivity) and hemodynamic acceleration may explain the changes observed in the electrocardiogram.

In mammals the occlusion operation has induced similar changes in respiration and circulation as observed in this study in the domestic fowl. In man a temporary occlusion of the pulmonary artery produces a slight hypoxia and hypercapnia (Grofman et al 1971). Rosenkrantz et al (1973) observed a 50 per cent increase in cardiac output after a 2-4 weeks occlusion period. Pulmonary hypertension, increase in the pulmonary resistance and hypoxia were observed after the chronic occlusion in pigs.

The morphology and morphometric features of normal lungs

of the domestic fowl in this study were similar to those reported e.g. by Weibel (1973). Osmiophilic inclusion bodies and surface lining have been found in the lungs of mammals (Kikkawa, Motoyama and Gluck 1968), reptiles (Dierichs 1973) and lung-fishes (Weibel 1973). In mammals these lamellar structures have been related to the production and transfer of the surfactant and the same has been proposed in avian species too (Tyler and Pangborn 1964). This has been opposed however by Jones and Radnor (1972 b). The morphological response to occlusion was a gradual loss of regular features. The distance from the air to the blood capillary increased. Osmiophilic lamellar inclusions decreased in number but were found. The surface membrane of the air capillary survived.

The infusion of the radiopaque substance to the aorta revealed that the main route for outflow of the bronchial blood is the pulmonary venous system. This bronchial vasculature is scarce in normal state. One week after occlusion a distinct increase in the systemic vascularity was observed. These vessels drained through the pulmonary veins. Thus the bronchial vasculature in the domestic fowl drains like in mammals and the response to pulmonary artery occlusion is similar to the mammalian response.

By histochemical methods Tyler and Pearce (1966) and Linsimies and Karkola (1973) have found the highest enzyme activities near the tertiary bronchi and the atria. Alkaline phosphatase and non-specific esterase reactions were very clear. These enzymes synthesize the surface active phospholipids. The enzymatic activities in the lung after occlusion operation first decrease and then increase during the active proliferative phase of growth of bronchial vasculature during the first post-operative weeks (Linsimies and Karkola 1973). The activity of naphthylamidase reaction indicated proliferative connective tissue during first two weeks after the occlusion. The activity of the succinate tetroxolium reductase indicates an altered metabolism in the lung with pulmonary artery occlusion and increased bronchial arterial flow.

The respiratory, circulatory and morphological response to the occlusion of the left pulmonary artery can be divided into three phases. The first acute stage with the reduction of the gas exchanging capacity is tolerated mainly by a reflectory process resembling the diving pattern. This response consisted of bradycardia, tachypnoea and probably bronchoconstriction.

The second stage, the beginning of adaptation, is mainly functional. Circulatory changes have the main part, although the adaptative changes in the blood cells are possible. At this stage the respiratory gas tensions normalize and the heart rate returns to control level, the contractile efficiency of the heart increases, as shown by the increased stroke volume and cardiac output and changes in the electrocardiogram.

During this stage of adaptation, morphological reorganization, the bronchial vasculature increases, enzyme activities become restabilized (Linsimies and Karkola 1973) and osmophilic substances are found. Thus, at least other functions of the lung than only respiration (temperature regulation, water evaporation etc) are possible (Lasiewski 1972). Whether this bronchial circulation is capable of gas exchange as in mammals, and what are the hematological adaptative features, remain to be seen in the further studies.

8 ABSTRACT

In order to study the capability of the gas exchange function and blood circulation to tolerate and adapt to the occlusion of the left pulmonary artery in the domestic fowl occlusion operations were performed in a series of 101 animals. The respiration rate, arterial and venous oxygen and carbon dioxide partial pressures and oxygen saturation, arterial blood pressure, heart rate, cardiac output, stroke volume, peripheral vascular resistance and electrocardiogram were registered. Morphology of the lungs was studied by histological, microangiographical and electromicroscopical methods.

The response could be divided into three stages. An acute response to the occlusion (from minutes to hours): tachypnoea with initial hypoxia and hypercapnia and bradycardia were found. This response resembled the diving pattern dominated by the increased vagal tone. Bradycardia and hypoxia returned to the control values in one day. Tachypnoea and elevated PaCO_2 were still evident after one week.

During the second stage, functional adaptation (from days to weeks), increases in cardiac output and stroke volume could be a sign of compensatory enhancement of the circulation and thus considered as induced adaptation process. Electrocardiographic changes, increased P-wave amplitude and QRS-duration further pointed to the temporary increased load of the right side of the heart and later to the improved effectivity of the left ventricle.

The third stage, morphological reorganisation (from week onwards) partly explains the increased output of the heart. The increase in the bronchial vasculature probably also indicated increased flow. The run-off of this new vasculature in the gas exchange zone was through pulmonary veins. In addition, the osmophilic structures and the epithelial surfactant were only slightly reduced. These morphological observations together with enzyme histochemical studies previously reported by the author indicate that the occluded lung still had largely its functional abilities.

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Maanantai, December 1974

Esko Länsmies

PS During the preparation of the manuscript the centenary anniversary of the birth of August Krogh (15 11 1874 - 14 9 1949) took place His life-long work on the comparative physiology of respiration is monumental and would deserve a lot more respect than just mention in this connection

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11 APPENDIX

Tables II - VIII

Figures IX - XXV

TABLE I Respiration rate P_{O_2} P_{CO_2} and pH of the blood of the domestic fowl

Reference	Year	Respiration rate breaths/min	pH	P_{CO_2} mmHg	P_{O_2} mmHg	Notes
Chiodi et al	1965	-	7.49	32.8	99.1	arterial
Richards et al	1967	27	7.47	-	95	anesthesia arterial
Burton et al	1968	26.4	7.51	34.6	109.9	male arterial
- -	-	31.0	-	-	-	female arterial
Pilpor et al	1970	23	-	29.2	87.0	arterial
- -	- -	23	-	39.3	40.8	venous
Bouverot et al	1972	-	7.55	24.5	89.5	capillary
Oshima et al	1974	38.5	-	-	-	male

TABLE II Blood pressure and heart rate in the domestic fowl

Reference	Year	Mean arterial pressure mmHg	Heart rate beats/min	Age	Sex
Woodbury <u>et al</u>	1937	161	-	?	?
Redbard <u>et al</u>	1946	100	-	?	?
Redbard <u>et al</u>	1947	125	-	Young	?
Sturkie <u>et al</u>	1953	131	328	Fullgrown	Female
Sturkie <u>et al</u>	1953	164	264	Fullgrown	Male
Ringer <u>et al</u>	1957	-	326	Young	Female
Ringer <u>et al</u>	1957	-	286	Young	Male
Sturkie <u>et al</u>	1959	142	378	Young	Female
Sturkie <u>et al</u>	1959	166	307	Young	Male
Zuckerman	1959	-	353	?	?
Vogel <u>et al</u>	1963	144	356	?	Female
Sturkie	1967	168	-	Fullgrown	Male
Burton <u>et al</u>	1968	-	350	?	Female
Sturkie <u>et al</u>	1970	-	303	?	Male
Piiper <u>et al</u>	1970	144	377	Young	Female
Oshima <u>et al</u>	1974	-	332	Young	Male

TABLE III Cardiac output stroke volume and peripheral arterial resistance in the domestic fowl

Reference	Year	Cardiac output ml/min/kg	Stroke volume ml/beat/kg	Peripheral resistance mmHg ml/sec	Method	Anesthesia	Sex
Sapirstein et al	1959	218	-	-	86RB-dilution	pento-barbitone	?
Sturkie et al	1959	143	0.49	31	Dye dilution	-	male
	-	173	0.46	29	-	-	female
Vogel et al	1963	240	0.67	22	-	-	-
Sturkie	1967	180	-	21	-	-	male
Pilper et al	1970	270	0.71	20	-	-	female

TABLE IV List of the 3-4 months old female animals used in the respiration studies. The identification number and weight (kg) of the animals are indicated. Recordings were made before and after the occlusion of the left pulmonary artery.

	No	Weight	Control	3	15	30	1 week	2 weeks
Control animals	75	1.6	x					
	76	1.6	x					
	78	1.5	x					
	81	1.7	x					
	87	1.7	x					
Sham-operated controls	93	1.0	x	x	x	x		
	94	1.1	x	x	x	x		
	95	1.2	x	x	x	x		
	96	1.2	x	x	x	x		
	97	1.1	x	x	x	x		
	98	1.2	x	x	x	x		
	99	1.0	x	x	x	x		
	100	1.2	x	x	x	x		
	101	1.3	x	x	x	x		
Examined for one week after occlusion	77	1.6	x	x	x	x	x	
	84	1.7	x	x	x	x	x	
	88	1.7	x	x	x	x	x	
	89	1.4	x	x	x	x	x	
	91	1.7	x	x	x	x	x	
Examined for two weeks after occlusion	79	1.7	x	x	x	x		x
	80	1.7	x	x	x	x		x
	82	1.7	x	x	x	x		x
	85	1.7	x	x	x	x		x
	86	1.7	x	x	x	x		x
	90	1.7	x	x	x	x		x

TABLE V List of the 3-4 months old female animals used in the hemodynamic studies. The identification number and weight (kg) are indicated

	No	weight	Control	3	15	30	1 week	2 weeks
Control animals	1	1.7	x					
	17	1.6	x					
	65	1.9	x					
	66	1.8	x					
	74	1.5	x					
	75	1.6	x					
	76	1.6	x					
	78	1.5	x					
	81	1.7	x					
	87	1.7	x					
Sham-operated controls	93	1.0	x	x	x	x		
	94	1.1	x	x	x	x		
	95	1.2	x	x	x	x		
	96	1.2	x	x	x	x		
	97	1.1	x	x	x	x		
	98	1.2	x	x	x	x		
	99	1.0	x	x	x	x		
	100	1.2	x	x	x	x		
	101	1.3	x	x	x	x		
Examined 15 min after occlusion	10	1.5	x		x			
	67	1.5	x		x			
	68	1.3	x		x			
	69	1.2	x		x			
	70	1.5	x		x			
Examined for one week after occlusion	64	1.3	x		x		x	
	71	1.4	x		x		x	
	72	1.5	x		x		x	
	73	1.4	x		x		x	
	77	1.6	x	x	x	x	x	
	84	1.7	x	x	x	x	x	
	85	1.7	x	x	x	x	x	
	89	1.4	x	x	x	x	x	
	91	1.7	x	x	x	x	x	
Examined for two weeks after occlusion	79	1.7	x	x	x	x		x
	80	1.7	x	x	x	x		x
	82	1.7	x	x	x	x		x
	85	1.7	x	x	x	x		x
	86	1.7	x	x	x	x		x
	90	1.7	x	x	x	x		x

TABLE VI List of the occlusion operated animals used in the electrocardiographical recordings Identification number and weight (kg) of the animal is indicated Registrations were performed before and after occlusion of the left pulmonary artery

No	Weight	Control	15	1-2 days	3 days	1 week	2 weeks
21	0 8	x	x				
27	0 9	x	x	x	x	x	
28	1 0	x	x	x	x	x	x
29	0 8	x	x	x	x		
31	1 7	x	x	x	x	x	x
33	1 7	x	x	x	x	x	x
36	2 0	x	x	x	x	x	x
39	1 8	x	x	x	x	x	x
40	1 8	x	x	x	x	x	x
41	2 0	x	x	x	x	x	x
42	2 0	x	x	x	x		
43	2 2	x	x	x	x	x	x
44	2 3	x	x	x	x	x	x
45	2 3	x	x	x	x	x	x
46	2 0	x	x	x	x	x	x
49	2 5	x	x	x	x	x	x
50	1 5	x	x	x	x	x	x
51	1 5	x	x	x	x	x	x
52	1 6	x	x	x	x	x	x
53	2 5	x	x	x	x	x	x
54	1 8	x	x	x	x	x	x
55	2 4	x	x	x	x	x	x
56	2 5	x	x	x	x	x	x

VIII

TABLE VII Electrocardiographical parameters (means in the domestic fowl before and after occlusion of the left pulmonary artery)

	Control	15	1 day	3 days	1 week	2 weeks
P-wave duration (msec)	19	21	20	20	20	19
PR-interval (msec)	67	80	69	72	72	70
QRS-duration (msec)	21	23	23	23	23	23
P-wave amplitude (mV)	0.047	0.062	0.108	0.094	0.073	0.065
Mean electrical axis (degrees)	-67	-54	-56	-55	-61	-72

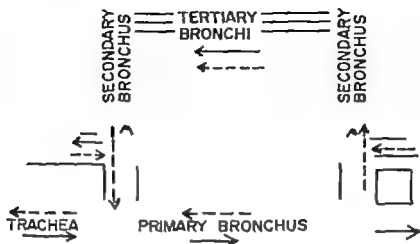


Fig. 1 Direction of the flow of air in the avian lung during spontaneous inspiration (—→) and expiration (---→)

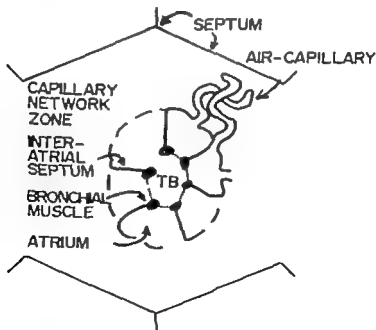


Fig. 2 Cross section of a tertiary unit TB = tertiary bronchus

FIGURE 3

Scheme of the gas-exchanging apparatus of the avian lung modified from Salt and Isuthen (1960) and Scheid and Piiper (1970). Arrows show the direction of blood and air

FIGURE 4

P_{O_2} in mmHg (e) and blood oxygen saturation in per cents (o) in arterial (—) and venous (---) blood (Mean \pm SEM) before and after unilateral occlusion of the pulmonary artery or sham operation (-) in the domestic fowl

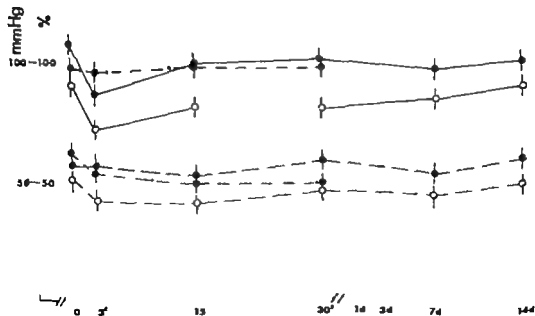
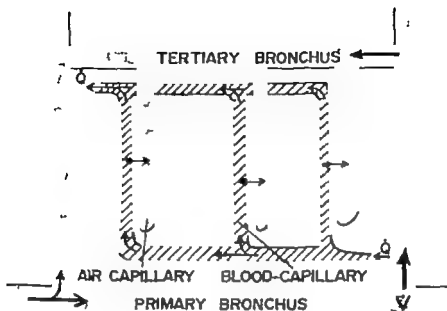


FIGURE 5

Respiration rate in breaths per minute (•)
 P_{CO_2} in mmHg (o) and pH (Δ) in arterial (—)
 and venous (---) blood (mean \pm SEM) before
 and after occlusion of the left pulmonary
 artery or sham operation (-) in the domestic
 fowl

FIGURE 6

Mean arterial pressure in mmHg (•) and heart
 rate in beats per minute in ECG-group (Δ) and
 hemodynamic group (o) sham-operated animals
 are indicated by a dotted line (- -)

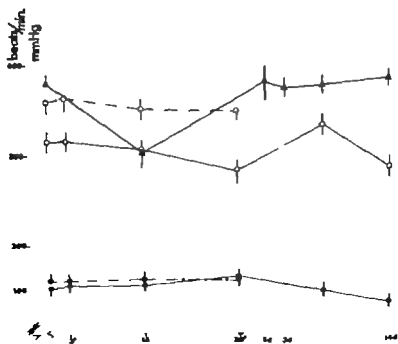
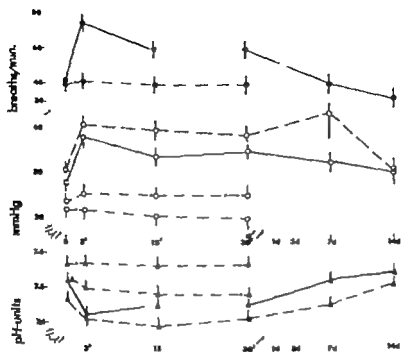


FIGURE 7

Arterial peripheral vascular resistance in PRU (o) cardiac output in ml/min/kg b w (e) stroke volume (□) in ml/beat per kg B w (mean \pm SEM) before and after unilateral occlusion of the pulmonary artery in the domestic fowl

FIGURE 8

Mean electrical axis in degrees (o) and P-wave amplitude in mV (x) (mean \pm SEM) before and after unilateral occlusion of the pulmonary artery in the domestic fowl

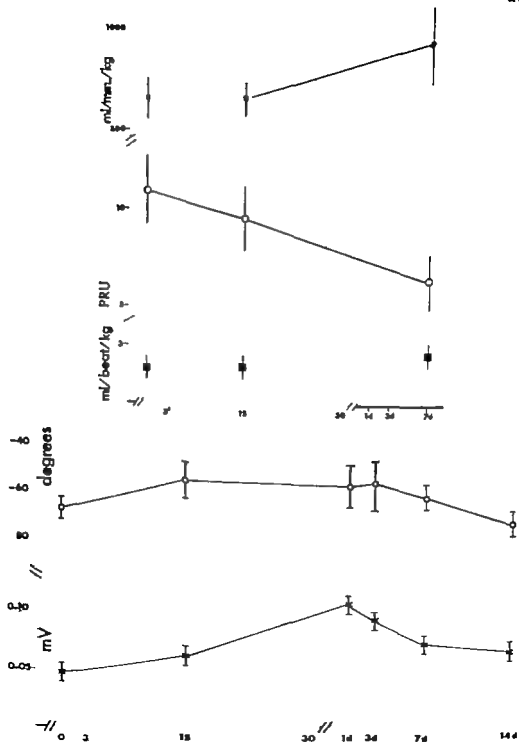


FIGURE 9

Left lung of a young control animal Verhoeff's elastic staining The scale is indicated in the figure (50 μ m) The tertiary Bronchus and atria are seen on the left side of the figure Smooth muscle ring has a diameter of 250 μ m Some of the atria are visible as open some as collapsed spaces Elastic fibers are seen in the inter-atrial walls In the middle of the figure a septum is seen with two arteries diameters about 70 μ m Light microscopy control lung

FIGURE 10

Right lung of the occlusion-operated young animal The scale is indicated in the figure (100 μ m) Several hexagonal lunglobuli are seen a poor filling of the septa a rather good filling of the inter-atrial walls (arrow) and the capillary network zone Microangiograph control lung injection through the aorta



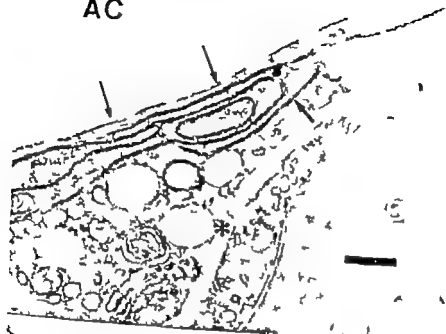
FIGURE 11

Young control animal the scale is indicated in the figure (0.5 μ m) A blood capillary with a red cell (BC) and an air capillary (AC) are visible The basal membrane (asterisk) is regular and thin Osmiophilic lamellar structures are shown with an arrow The thin surface lining of the air capillary is indicated with small arrows
Electron micrograph control lung

FIGURE 12

Explanations as in Fig 9 note the dual osmiophilic lamina on the air-capillary surface (small arrows)
Electron micrograph control lung

AC



AC



FIGURE 13

Left lung of a young female chicken occlusion-operated one week earlier. The scale is indicated in the figure ($100\text{ }\mu\text{m}$): Centrifugal distribution of the radiopaque medium. Septal venules and filling of the capillary network zone. Note the collection of the contrast medium near the tertiary airway (arrow).
Microangiograph occluded lung injection through the aorta

FIGURE 14

Left lung of a young chicken with left pulmonary artery occluded one week earlier. The scale is $100\text{ }\mu\text{m}$. Late stage of contrast filling of the veins. Note the millipede-like configuration (arrow) of the pulmonary venule. The asterisk indicates an enlarged bronchial arteriole.
Microangiograph occluded lung injection through the aorta

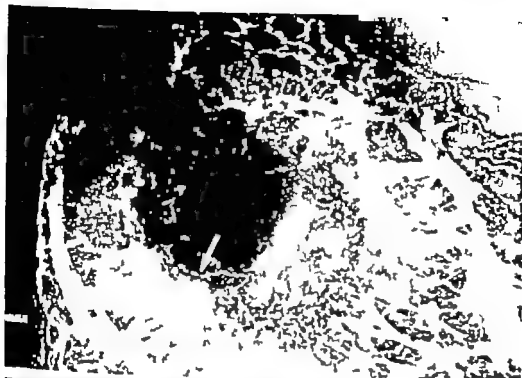


FIGURE 15

Left lung after the 12 days post-occlusion period in a young chicken Verhoff's elastic staining the scale is 50 μ m The connective tissue in the septum is increased Two tertiary bronchioli are visible One blood vessel in the capillary network zone is indicated by an arrow Light microscopy occluded lung

FIGURE 16

The left lung three months after occlusion of the pulmonary artery in a young animal (Verhoeff's staining the scale is 50 μ m) Air capillaries of various sizes are seen Small vessels are seen scattered among the air capillaries but distinctly apart from them Light microscopy occluded lung

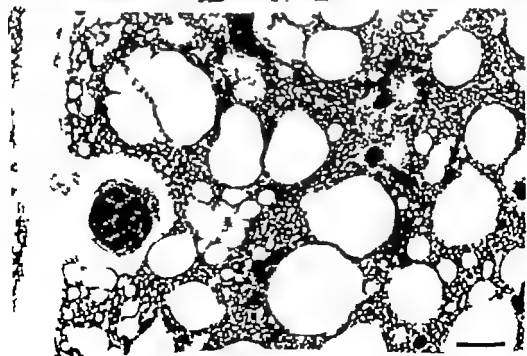
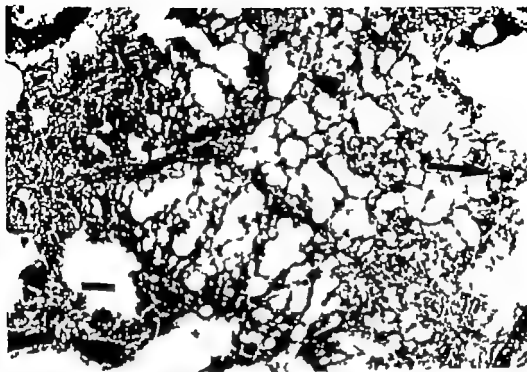


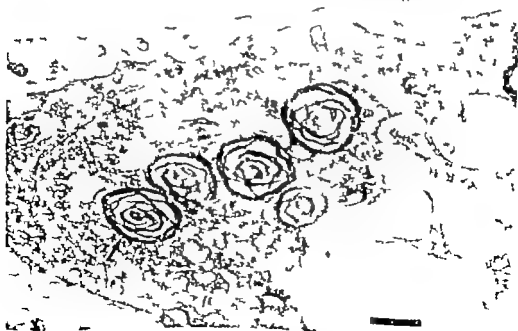
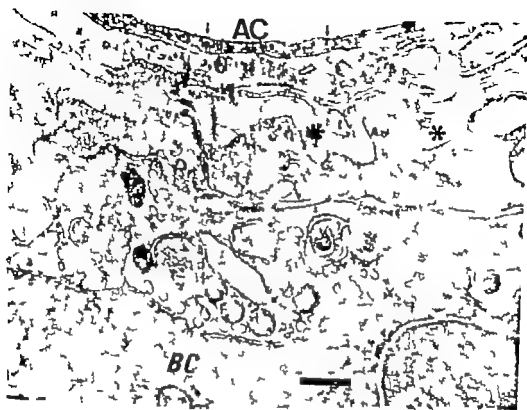
FIGURE 17

Electron micrograph of the left lung four months after occlusion of the left pulmonary artery (scale 0.5 μ m). Note the tortuous basal membrane and the long distance from the air capillaries to the blood capillaries. The osmiophilic lining of the air capillary is visible (arrows).

Electron micrograph occluded lung

FIGURE 18

The left lung of a young chicken four months after occlusion of the left pulmonary artery (scale 0.5 μ m). The arrow shows one lamellar osmiophilic inclusion. Electron micrograph occluded lung



ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 423

**OSMOLAR CONTROL
OF THE CIRCULATION
IN HEMORRHAGIC HYPOTENSION**

An experimental study in the cat

By

Johannes Jarhult

Lund 1975

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 423

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OSMOLAR CONTROL OF THE CIRCULATION IN

HEMORRHAGIC HYPOTENSION

An experimental study in the cat

By

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This thesis is based mainly on the following publications

- I JARHULT J J LUNDVALL S MELLANDER and S TIBBLIN Osmolar control of plasma volume during hemorrhagic hypotension Acta physiol scand 1972 85 142-144
- II JARHULT J Osmotic fluid transfer from tissue to blood during hemorrhagic hypotension Acta physiol scand 1973 89 213-226
- III JARHULT J and P-O GRANDE Transcapillary fluid movements in sympathectomized intestine and skin during hemorrhagic hypotension Acta physiol scand 1975 In press
- IV JARHULT J Role of the sympatho-adrenal system in hemorrhagic hyperglycemia Acta physiol scand 1975 93 25-33
- V JARHULT J and S MELLANDER Autoregulation of capillary hydrostatic pressure in skeletal muscle during regional arterial hypo- and hypertension Acta physiol scand 1974 91 32-41
- VI JARHULT J J HILLMAN and S MELLANDER Circulatory effects evoked by physiological increases of arterial osmolality Acta physiol scand 1975 93 129-134

In the text these papers are referred to by their Roman numerals

INTRODUCTION

Hemorrhage implies a stress situation for the organism which is met by compensatory mechanisms that often are capable of restoring cardiovascular homeostasis. If the compensation is insufficient and in particular if secondary circulatory derangements or other complications occur hemorrhagic shock may finally ensue. Various aspects of the latter topic have been reviewed previously for instance by Wiggers (1950), Sealey and Weisiger (1961), Bock (1962), Hershey (1964), Fine (1965), Mills and Moyer (1965) and Weil and Shubin (1967).

The present study deals with compensatory adjustments occurring in early stages of hemorrhage with particular reference to the development of plasma hyperosmolality and its role in cardiovascular control.

The defence mechanisms in bleeding are primarily aimed at limiting the deleterious effects of hypovolemia on tissue perfusion and nutrition. The rapid augmentation of sympathetic discharge leading to improved cardiac performance, increased peripheral resistance and constriction of the capacitance vessels constitutes an important first line of defence but can cause no definite restoration. The adequate compensation of course implies a replacement of the shed blood volume. The restitution of the red cell volume is however a slow physiological process and therefore the refill of the circulatory system in the early stages of hemorrhage must be accomplished by an increased plasma volume.

Previous studies have revealed different mechanisms for such restoration of plasma volume in hypovolemia. Most important perhaps is a transcapillary absorption of interstitial fluid mediated by an adrenergic reflex resetting of the pre-/postcapillary resistance ratio which lowers hydrostatic capillary pressure in muscle and skin tissues (Uberg 1964). More gradual

adjustments are accomplished by the endocrine and reflex regulation of renal water and salt excretion (cf Wiggers 1950 Laragh and Sealey 1973) and by control of the fluid intake via the thirst mechanism (cf Fitzsimons 1972)

The present investigation in cats indicated that in hemorrhage there is also an important osmolar control of plasma volume related to substances other than the plasma proteins. In fact this regulatory mechanism seems primarily aimed at an overall restoration of extracellular fluid volume effected by osmotic fluid withdrawal from the intracellular space. A similar control principle was recently shown to be operating in heavy exercise (Lundvall Hellander Westling and White 1972).

It will be demonstrated that hemorrhagic hypotension is associated with a pronounced increase of blood osmolality (Chapter 3) in turn mainly caused by glucose release from the liver (Chapter 4). The reflex control mechanisms for this glucose liberation are described in Chapter 5. That the blood borne hyperosmolality leads to a transcapillary osmotic absorption of extravascular fluid into the blood stream is shown in Chapter 6 and this process occurs mainly in skeletal muscle and skin tissues. From a quantitative point of view the osmolar plasma volume control seems to be as important as the previously known adrenergic reflex mechanism at least in severe hemorrhage. Plasma volume expansion by fluid absorption does not result from the arterial hypotension per se since as described in Chapter 7 the expected passive fall of capillary hydrostatic pressure is effectively prevented by local vascular autoregulatory mechanisms. Finally it is shown that the hemorrhagic hyperosmolality exerts some inhibitory action on the tone of the resistance vessels in several tissues and concomitantly a moderate positive inotropic action on the heart (Chapter 8).

Chapter 2

METHODOLOGY

Material and anesthesia

Experiments were performed on a total of 143 cats of both sexes (mean weight 3.2 kg) anesthetized intravenously with a halothane (50 mg/kg b.wt) after induction with ether. The majority of the cats also received an initial small dose of pentobarbital sodium (5-20 mg). In some pilot experiments the anesthesia was supplemented with urethane (100 mg/kg b.wt). The animals were kept on a normal mixed diet and fasted for about 16 hr before the experiments but received water ad libitum.

Surgical procedures

Vascular reactions were studied in four different tissues: skeletal muscle, kidney, intestine and kidney.

Skeletal muscle preparation. Studies were performed on the lower leg muscles (below called calf muscles) in 81 cats and the legion was prepared according to the technique described by Kjellme (1964). In brief the skin of the right hind leg was dissected free from the underlying muscle tissues down to the ankle and the paw removed at the ankle joint. The thigh muscles were separated from the lower leg. The bone marrow cavity of the distal femur was drilled open and plugged with cotton soaked in silicone grease to occlude intra-osseous vascular connections. Small vessels between the thigh and lower leg were ligated so that the popliteal artery and vein formed the sole vascular connection with the main part of the body. With few exceptions (11 V) the musculoperitoneum was acutely sympathectomized by cutting the sciatic nerve. The popliteal vein was cannulated and the outflow directed to the external jugular vein in an optical drop recorder unit. When changes in tissue volume were to be recorded the musculoperitoneum was enclosed in a water-filled temperature controlled (38°C) plethysmograph along the kidney flap for proximal perfusion.

In some experiments (11 V) the total blood flow to the calf muscles was diverted from the proximal femoral artery by a T-tube, inserted into the popliteal artery to permit local perfusion of drug and precise measurements of arterial blood flow pressure. In 9 of these experiments (11) a perfusion pump (Harvard model 1210) was inserted into the T-tube to allow

adjustments of the regional blood flow to desired levels. In the hypotension experiments described in page V the regional arterial inflow pressure was manually adjusted by a screw clamp proximal to the T tube.

Skin preparation The hind paw was chosen for studies of the circulatory events in a cutaneous vascular bed (20 cats). To observe reactions mainly in the cutaneous skin vessels the pads (with their abundant arterio-venous anastomoses) were excluded from the circulation by tight ligatures. The skin of the calf was dissected free from the knee down to the ankle joint. All superficial and deep veins were ligated 1-2 cm above the joint except for the great saphenous vein. This vessel was cannulated and its outflow directed to the jugular vein. In an optical drop recorder. In some experiments (VI) drainage through deep veins was prevented by an ankle cuff the pressure in which was raised slightly above venous outflow pressure. When desired the paw was sympathectomized by severing the sciatic and the femoral nerves. To record changes in tissue volume (III) the paw was placed in a perspex plethysmograph filled with water using the calf skin flap for proximal closure. The water temperature was kept at 34°C (neutral temperature). In two experiments (III) the circulatory events in the paw were studied during regional hypotension. For this purpose a short shunt circuit was inserted between the femoral and popliteal artery, all other arterial vessels between the thigh and the calf being ligated. Regional arterial pressure was then monitored from a T tube in the shunt and could be adjusted to desired levels by a screw clamp.

Intestinal preparation In 16 cats without signs of intestinal infection a preparation of the small intestine was made the principle of which has been described by Folkow et al. (1963). In brief a segment of the jejunum (weighing 15-18 g) was isolated and the mesenteric of the intestines removed. With some exception (VI) the regional autonomic nerves were ligated and cut. The venous outflow from the segment and its lymph node was measured with an optical drop recorder and returned to the animal via the external jugular vein. To record changes in tissue volume (III) the intestinal preparation was enclosed in a perspex temperature controlled (38°C) plethysmograph filled with Tyrode solution. A short distal shunt circuit was inserted in the splanchnic artery in these plethysmographic experiments. Closure of the plethysmograph at the site of the entrance of the two cognate vessels was accomplished with silicone grease.

Kidney Blood flow from the left kidney was recorded in 8 experiments (VI). After ligation of the left spermatic or oviductal the main renal vein was cannulated and the venous outflow directed to an optical drop re-

corder to the external jugular vein. The regional sympathetic nerves were ligated and cut in 3 of these experiments.

Interference with the sympathoadrenal system. Observations of vascular reactions were made in the different regions both with intact sympathetic innervation and after regional sympathectomy. In the latter experiments phenoxybenzamine (1.4 mg) was given close arterially to prevent an influence from circulating catecholamines. Significant systemic effect of the blocking drug was avoided by collecting and discarding the venous outflow for about 3 min after the injection. This volume of blood was substituted by the same amount of isotonic dextran Tyrode solution. The effectiveness of the α blockade was checked by close arterial injection of norepinephrine (5-10 $\mu\text{g/kg}$ b.wt.).

In paper IV special attention was paid to the influence of different links in the sympathoadrenal system on the glucose release from the liver during hemorrhage. This analysis was performed on 33 cats divided into 4 different groups. In 14 cats the sympathoadrenal system was left intact. In 7 cats the adrenal gland was carefully removed; in 6 cats the hepatic nerve plexus was dissected free around the hepatic artery and portal vein, ligated and cut. In 6 cats the major and minor splanchnic nerves were avulsed bilaterally and in addition both adrenals removed.

Catheterization techniques for blood sampling. Before catheterization the animals were heparinized (750-1000 IU/kg b.wt.). Most of the present experiments blood samples were taken for determinations of plasma osmolality and plasma glucose concentration. Arterial samples were withdrawn from catheters in the right carotid artery and venous samples from the outflow catheters in the four different regions (see above). In paper IV an analysis was made of the role of the liver in the hemodynamic hyperglycemic response by determining glucose, osmolality and glycogen level in plasma from blood samples in the venous system. For this purpose two fine cannulized polyethylene catheters were inserted into the inferior caval vein of the two major saphenous veins. One of these was advanced until its tip was positioned just above the iliac bifurcation (below called iliac vein) and the other one just above the entrance of the hepatic vein (below called hepatic vein). In some of the experiments ($n=7$) a catheter was also inserted into the portal vein via cannulation of a distal branch of the superior mesenteric vein.

Cardiovascular recording

Mean (and sometime pulsatile) arterial blood pressure was measured with

Statham P23 AC transducers placed at heart level. The pressure was monitored from the right carotid artery or (in paper III) from the left femoral artery. Regional venous outflow pressures were determined from the height of the outflow tubing above heart level or sometimes (V) measured directly with a Statham P23 DC transducer from a T tube placed close to the cannula in the popliteal vein. The transducer was then positioned at the water level in the plethysmograph to compensate for the induced external water pressure. In some of the hypotension experiments (V) the pressures in a small muscle artery (SAP) and a small muscle vein (SVP) were monitored from drawn-out flexible nylon tubings inserted in the sural artery and vein and recorded with Statham transducers (for details see Haddy et al 1954, Lundvall 1972).

Heart rate was recorded with a Grass tachograph triggered by the systolic pressure wave (VI).

The regional blood flows were continuously registered with silicone filled optical drop recorder units inserted on the venous side (Lindgren 1958).

Changes of the tissue volume were recorded with the plethysmographic technique originally described by Mellander (1960). The plethysmograph design was modified to fit the various tissues. In some early experiments (I-III) changes of tissue volume were registered with a piston recorder and later (III-V) with an electronic gasimetric transducer (Grass FT 10; for details see G. Jönke et al 1974).

The cardiovascular parameters were usually recorded on a Grass polygraph or occasionally on a kymograph.

Analysis of peripheral vascular function

Regional vascular resistance was calculated from the arterio-venous pressure gradient and regional blood flow and expressed as mm Hg/(ml/min \times 100 g tissue).

Data on capacitance responses and intercapillary fluid movements were deduced from the observed changes of tissue volume according to the principle described by Mellander (1960). This implies that abrupt changes of tissue volume coordinated in time with changes of vascular resistance are considered to reflect the responses of the capacitance vessels whereas gradual changes of tissue volume occurring in face of a maintained steady state resistance response are considered to represent net intercapillary fluid movements. This interpretation of the volume curve has been validated

In vivo studies by the simultaneous use of radioactive tracer techniques (e.g. Åblad and Mellander 1963, Kjällmer 1965)

Reaction of the precapillary sphincters were estimated with the capillary filtration coefficient (CFC) method (Cobbold et al 1963, Folkow and Mellander 1970). The venous pressure increase applied during determinations of the CFC was less than 5 mm Hg. In these experiments to minimize myogenic reaction (cf. Mellander et al 1964) unless otherwise stated a ratio of pre-/postcapillary resistance of 4/1 was used for the CFC calculations (Pappenheimer and Soto-Rivero 1948).

Soft tissue weight of the studied region was determined at the end of the experiment.

Spread of data is given as the standard error of the mean. Significance tests were performed according to Student's t test.

Biochemical analyses

Plasma osmolality was determined with thermal conductivity (Osmometer 31 LAS, Advanced Instruments Inc.). Each sample was measured twice and the mean value used. Repetitive reading on osmometer did not deviated more than 1.5 mOsm/kg H_2O from the true value.

Plasma glucose concentration was determined by the glucose-oxidase technique.

Plasma glucagon concentration (immunoreactive glucagon) was estimated with a radioimmunoassay technique developed by Nilsson and Wilensten (1975).

Plasma sodium and potassium concentration was determined with flame photometry.

Plasma urea concentration was determined with the Technicon Autoanalyzer System.

Experimental procedure

Hemorrhagic hypotension experiments The animal rested for about 30 min before the completion of the surgical preparation. At the end of this control period 10-15 ml venous blood samples were withdrawn for biochemical determinations. In the plethysmographic studies (I III V) venous outflow pressure in the different region was set so as to establish approximately an isovolumetric state in the control situation (Starling equilibrium).

Hemorrhagic hypotension was achieved by bleeding the animal to 10-15

canalized pressure bottle connected via a T tube to the right carotid artery. The exsanguination was rapid so that arterial blood pressure reached a level of 50 mm Hg within 3 min. This pressure level was then maintained throughout the experiment with the aid of the pressure bottle. Changes of the peripheral vascular functions in the different regions were followed during the hypotensive period which in most cases lasted for 90 min. Repetitive arterial and venous blood samples were taken for biochemical analyses during the period of hypotension. The animals breathed spontaneously throughout the experiments.

Some comments will be given here concerning the above-mentioned experimental hemorrhage model. This model was originally developed in Wiggers laboratory (Verbe et al. 1942) and has later been extensively used. It implies that the animal is kept at a constant hypotensive level irrespective of compensatory adjustments. The model has several advantages. For instance, it permits detailed studies of net transcapillary fluid movements without interference from a fluctuating arterial inflow pressure and venous outflow pressure which aided in the interpretation of the present results. The analysis of capillary fluid transfer during bleeding was furthermore facilitated in the present experiments by blockade of the lymph drainage from the studied regions. It should be made clear, however, that this bleeding model creates an artificial hemorrhagic situation insofar that the animal cannot benefit from the effects of the compensatory mechanisms that normally tend to restore blood pressure and volume. Animals with vivid sympatho-adrenal reflexes will therefore be exposed to a more intense vasoconstriction and hence to greater blood loss than those with less effective reflex mechanisms (cf. Chien 1967). This was noticed in the present experiments in terms of a relatively wide range of the volume of shed blood. Thus, the fraction of total blood volume (assumed to be 7 per cent of the body weight in the control situation, see Altman and Dittmar 1971) which had to be withdrawn initially to lower blood pressure to 50 mm Hg varied in the different animals from 11.35 per cent (mean value $26 \pm 2\%$).

Cardiovascular and biochemical analyses were also performed in a few hemo-oedematous animals prepared as described above for the skeletal muscle region. These animals were not bled after the initial control period but left undisturbed for 90 min.

Regional hypotension experiment. The transition from normo- to hypotension in the hemorrhagic experiments causes a marked change of the perfusion gradient and the transmural pressure in the peripheral vascular beds. To analyse possible local effects of such pressure alterations per se, re-

gional hypotension experiments were performed (III V) in the following way. The above-mentioned vascular functions were observed in response to regional arterial hypotension of varying duration accomplished by distal occlusion of a screw clamp placed on the cognate artery to the region (skeletal muscle and paw). Vascular resistance and CFC were determined in the isovolumetric control period of normotension and the effects on vascular resistance, capacitance, CFC and net transcapillary fluid transfer were followed during periods of graded regional arterial hypotension (eng down to 30 mm Hg). In one series of these experiments arterial pressure in the muscle region was kept at 50 mm Hg for a period of 90 min and arterial and regional venous plasma osmolality was determined at intervals. In some other experiments papaverine (0.6-1.6 mg/min) was infused close arterially to abolish vascular tone and hence active local responses to the regional hypotension.

An analysis of local vascular effects in skeletal muscle was also performed during short-term periods of arterial hypotension up to 170 mm Hg. The hypotension was evoked by bilateral carotid occlusion after cervical vagotomy. Alpha-adrenergic effects in the studied muscle region were then prevented by regional sympathectomy and by selective distal administration of phenoxybenzamine (10 mg/kg muscle tissue).

Hypertonic infusion experiments. As will be shown, hemorrhage leads to an increase of the plasma osmolality. In an attempt to study selectively the effects of plasma hyperosmolality per se on cardiovascular function, hypertonic solutions were infused intravenously to existing non-bled animals at a rate which increased the arterial osmolality to about the same levels as in the present hemorrhage experiment. Hypertonic (30-50 %) solutions of glucose or saccharose were then slowly infused in the right axillary vein at a rate of 0.3-1.0 ml/min. In one series of these experiments (VI) the effect on vascular resistance in skeletal muscle, kidney and on mean and pulsatile arterial pressure and heart rate was observed. The hypertonic infusion lasted for 5 min and were repeated at intervals 2-3 times in each animal. The cardiovascular parameter and arterial plasma osmolality were analyzed before and during and for 15 min after the hypertonic infusion. In other series of hypertonic infusion experiments (Chapter 6 and paper III) the effect on net transcapillary fluid movement in the sympathectomized skeletal muscle paw and intestine was observed. Hypertonic glucose (30 %) was slowly infused for 10-30 min in these experiments and arterial and regional venous osmolality determined at intervals. The adrenals were removed in these experiments to exclude effect of possible release of catecholamine.

Chapter 3

CHANGE OF PLASMA OSMOLALITY DURING HEMORRHAGIC HYPOTENSION

(Paper I-IV)

General considerations

Little attention has been paid to the problem of osmolar changes in hemorrhage. In 1963 Brooks et al. reported that the arterial plasma osmolality in dogs was virtually unchanged after 90 min of hemorrhagic hypotension at a level of 40 mm Hg. They found however an increase of the plasma glucose concentration and explained the plasma isotonicity by an equi-osmolar drop of the plasma sodium concentration. Bergentz and Brief (1965) on the other hand demonstrated that severe hemorrhage in dogs in fact could be associated with a considerable increase of the arterial plasma osmolality in their experiments amounting to about 25 mOsm/kg H_2O after 20 min of arterial hypotension at 20 mm Hg. This finding in dogs was confirmed some years later by Baue et al. (1967), Boyd and Mansberger (1968) and Grega et al. (1971) although in the latter study the hyperosmolality was more moderate. Boyd and Mansberger revealed a hyperosmolar state also in humans in hemorrhagic shock.

The patho-physiological significance of the hemorrhagic hyperosmolality is poorly understood. Boyd and Mansberger (1968) considered the hyperosmolality in dogs and patients to be a bad prognostic sign insofar that they found an impaired survival in individuals with marked plasma hypertonicity in hemorrhagic shock. On the other hand Brooks et al. (1963), Bergentz and Brief (1965) and Baue et al. (1967) found that the survival rate in dogs was improved if the animals received a hypertonic instead of an isotonic infusion in the oligemic phase of hemorrhagic hypotension. The mechanism for such a beneficial effect however could not be revealed in these studies.

The hypothesis is advanced that the organism might benefit from the plasma hyperosmolality during hemorrhage insofar that it might cause an osmotic fluid absorption from the extravascular to the intravascular space with a consequent increase of plasma volume. Such a mechanism has recently been shown to be operating in heavy exercise (Lundvall et al 1972). An osmotic withdrawal of tissue fluid in bleeding would be possible however only if the plasma osmolality exceeds the tissue osmolality i.e. if the hyperosmolality is primarily blood borne. As a first approach to this problem detailed studies of arterial and venous plasma osmolality were performed during hemorrhagic hypotension.

Present results

Concomitant determinations of arterial and venous plasma osmolality during hemorrhagic hypotension at 50 mm Hg were made in 73 cats with intact sympatho-adrenal system (1 IV and unpublished experiments). Arterial samples were always taken from the carotid artery whereas the venous samples represented different tissues viz skeletal muscle and skin intestine kidney and liver.

Fig. 1 shows collected data from 63 cats on the osmolar changes in a skeletal muscle-skin region. The venous samples in this series were taken from different sites: popliteal vein (n=36), femoral vein (n=9), great saphenous vein (n=8) and iliac vein (n=10). The osmolality changes at these various sites in the venous system were not significantly different from each other and the data have therefore been pooled (Fig. 1). In the control period, arterial plasma osmolality averaged 319 ± 0.7 and venous plasma osmolality 321 ± 0.7 mOsm/kg H_2O (in agreement with Lundvall 1972). It can be seen from Fig. 1 that hemorrhage caused a marked increase of the arterial plasma osmolality with the most rapid change in the initial 10 min of hypotension. A clearcut increase could be detected as early as 45 sec after the start of

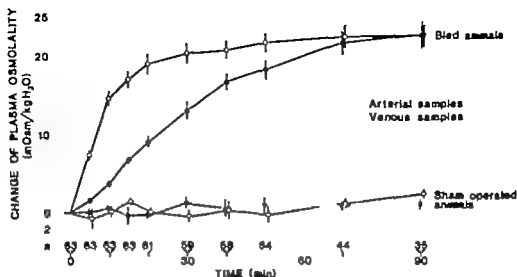


FIG 1 Changes from control value of arterial and popliteal venous plasma osmolality (mean values \pm S.E.) in cats bled to 50 mm Hg ($n=6$) and in sham operated cats ($n=4$)

the bleeding. The arterial hyperosmolality reached a value of about 20 mOsm/kg H₂O above control after 20 min, a level then maintained throughout the 90 min period of observation. Venous plasma osmolality on the other hand rose more gradually and did not reach the arterial level until some 60 min after the start of the hemorrhagic hypotension. A positive arterio-venous osmolar difference (implying a transcapillary osmolar gradient, see below) was thus present during the first hour, though most pronounced during the initial 30 min of hypotension. The sham-operated animals did not show any significant alteration of arterial or venous osmolality. In some experiments (1) the changes of arterial and venous plasma osmolality were observed during a more prolonged (3 hr) period of hemorrhagic hypotension at 50 mm Hg. These experiments showed that the hyperosmolality remained approximately constant at the high level throughout this period. During the last hour venous osmolality tended to slightly exceed arterial osmolality.

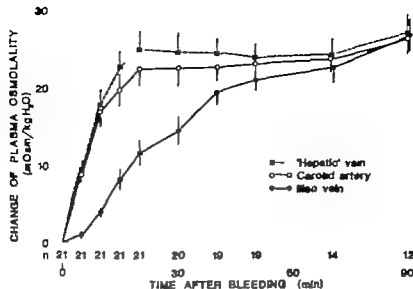


Fig 2: Simultaneously determined plasma osmolality of the hepatic and renal and iliac venous blood during hemorrhagic hypotension to 50 mm Hg for 90 min. Mean values \pm SE given.

Data for the intestinal ($n=8$) and renal ($n=2$) circulations showed that the osmolality pattern was basically similar to that described in Fig 1 insofar that the arterial osmolality was higher than the regional venous osmolality in the initial period of hypotension. In these tissues however venous osmolality approached arterial osmolality much earlier than in skeletal muscle and skin viz after 30 min in the intestine and within less than 10 min in the kidney. The difference can be ascribed to the much higher blood flow in intestine and kidney than in muscle.

The osmolar changes in venous samples from the hepatic vein (see Methodology) were distinctly different from those taken from the tissues mentioned. From the very beginning of the bleeding the hepatic venous osmolality thus exceeded the concomitantly determined arterial osmolality and was much higher than that in the venous blood from the hindquarters (Fig 2). On the other hand the degree of hyperosmolality in the portal vein ($n=7$)

was much smaller than in the hepatic vein

It was concluded that the hemorrhagic hyperosmolality to a significant extent was caused by release of some substance(s) from the liver. It should be stressed however that the hepatic venous blood was sampled in the inferior caval vein at the entrance of the hepatic vein thus implying considerable admixture of blood from regions other than the liver. The magnitude of the true hepatic venous hyperosmolality therefore must have been underestimated in Fig. 2

The data presented above refer to averages. Mention should be made that 6 animals out of the 73 did not respond with significant blood hyperosmolality during the hemorrhagic hypotension.

Comments

The present study has defined in detail the magnitude and the time course of the osmolar changes occurring in the arterial blood and in the venous effluent from several different tissues during hemorrhagic hypotension at 50 mm Hg. In the majority of the cats (92 %) hemorrhage evoked a rapid pronounced ($20 \text{ mOsm/kg H}_2\text{O}$) and maintained increase of arterial osmolality a finding in accordance with some previous observations in other species including man (Bergentz and Brief 1965, Baue et al 1967, Boyd and Mansberger 1968, Alali 1970, Boyd et al 1970). Our analysis indicated in addition that the hemorrhagic hyperosmolality to a major extent was caused by release of some osmotically active product(s) from the liver. The nature of this substance will be described in Chapter 4.

This investigation was made under a standardized type of hemorrhage. Preliminary experiments indicated that the magnitude of the hyperosmolar response is related to the severity of the bleeding which seems to explain some discrepancies reported in the previous literature (see above).

The patho-physiological significance of the hemorrhagic blood hyperosmo-

lality may be severalfold. As previously reported (Almli 1970) it might affect the fluid balance in the body via an influence on the thirst mechanism. It also seems to affect the internal fluid balance by causing a redistribution of fluid from the intracellular to the interstitial and intravascular compartments (Chapter 6). Hemorrhagic hyperosmolality might also influence cardiac function and peripheral vascular resistance (Chapter 8).

Chapter 4

RELATION BETWEEN HYPEROSMOLALITY AND HYPERGLYCEMIA IN HEMORRHAGIC HYPOTENSION (Paper II IV)

General considerations

In 1877 Claude Bernard discovered that the glucose concentration in the blood rose in dogs subjected to hemorrhage a finding later repeatedly confirmed in several species (see below). This suggested to us that glucose might be responsible to a significant extent for the hemorrhagic hyperosmolality described in the previous chapter and this hypothesis was tested in the present series of experiments.

Present results

From a few pilot experiments presented in Table I it became evident that the hemorrhagic plasma hyperosmolality indeed was mainly caused by an increased glucose concentration. Thus it can be seen that the gradual rise of plasma osmolality which after 70 min of hypotension exceeded the control value by $31 \text{ mOsm/kg H}_2\text{O}$ almost entirely could be attributed to the concomitant hyperglycemia. The potassium and urea concentrations also rose but the osmolar effect of these changes was more than counterbalanced by the decrease in sodium concentration. Calculated values for the total osmolar change induced by these agents (neglecting the osmotic activity coefficients) are given at bottom of the table. These values agreed relatively well with the observed osmolar increase except at 70 min when the observed osmolar change somewhat exceeded the calculated value.

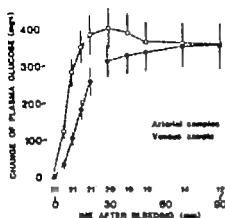
A more thorough investigation of the relation between the hyperosmolality and the hyperglycemia responses was made in 21 cats during hemorrhagic

TABLE 1 Changes of osmolality glucose sodium potassium and urea-N in arterial plasma during hemorrhagic hypotension at 50 mm Hg (mean values from 2 cats) The approximate total change of osmolality due to glucose sodium potassium and urea-N is depicted at bottom

	Control	Time of hypotension at 50 mm Hg		
		10 min	30 min	70 min
Osmolality (mOsm/kg H ₂ O)	325.0	337.8 +12.0	344.5 +19.5	356.0 +31.0
Glucose (mM/L)	15.0	26.6 +11.6	38.9 +23.9	41.1 +26.1
Sodium (mEq/L)	152.0	147.0 -5.0	145.5 -6.5	144.5 -7.5
Potassium (Eq/L)	3.5	6.7 +3.2	6.0 +2.5	8.5 +5.0
Urea-N (mM/L)	3.3	3.6 +0.3	3.9 +0.6	4.1 0.8
Calculated osmolality change (mOsm/kg H ₂ O)		+10.1	20.5	24.4

hypotension at 50 mm Hg of 90 min duration (Fig. 3). In the control period before bleeding arterial plasma glucose concentration averaged 220 ± 15 mg% and venous plasma glucose concentration 210 ± 14 mg% in blood drained from the hindquarters i.e. mainly representing skeletal muscle and skin tissues. It can be seen from Fig. 3 A that the arterial glucose concentration rose quickly and reached a level of 400 mg% above control after 30 min of hypotension. After this there was a slight gradual decline to about 350 mg% above control 90 min after the start of the bleeding. The venous plasma glucose concentration increased more slowly and did not reach the arterial level until about 1 hour after the commencement of the hypotension. The clearcut difference between the arterial and venous plasma glucose con-

A



B

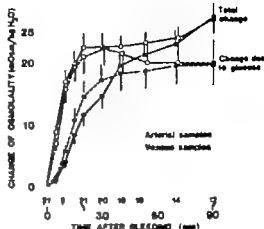


FIG. 3 **A** Changes from control value of arterial and popliteal venous plasma glucose concentration in cats bled to 50 mm Hg (n 21) **B** Changes above control value of total arterial and venous plasma osmolality in cats bled to 50 mm Hg (n 21) shown together with deduced data for osmolality changes caused by concomitant alteration of plasma glucose concentration. Mean values \pm S.E. given.

concentrations in the early stage of hemorrhage indicates that glucose entered the extravascular space in this period (cf Fig 3 paper II)

Fig 3 B shows the extent to which the hyperglycemia contributed to the concomitantly observed hyperosmolality in this material. For this deduction 18 mg% of glucose was considered to correspond to 1 mOsm/kg H₂O. It can be seen that a close correlation indeed existed between the observed changes of osmolality and the values deduced from the changed glucose concentration both in arterial and venous plasma. During the initial 30 min of hemorrhagic hypotension the hyperglycemia in fact seemed to almost fully account for the plasma hyperosmolality. In later stages of bleeding however the observed values for the total osmolality change clearly exceeded those calculated merely from the glucose change indicating that some other substance(s) contributed to the hyperosmolality at that time.

It should be emphasized here that all data on the glucose content refer to the concentration in plasma (mg/100 ml plasma) and not in whole blood the latter unit often being used in clinical work. In the control period before bleeding the plasma glucose level was about 215 mg% in these experiments which corresponds to a blood glucose level of 130 mg% taking the appropriate hematocrit value into account (and confirmed by direct determinations of the blood glucose level).

Comments

It is a well documented fact that hemorrhage is associated with hyperglycemia. This has been established in several species including man (for key references see Table II). It may be noted from the data in the table that the magnitude of the hyperglycemia response seems related to the severity of the hemorrhage a phenomenon which has been analysed in some detail previously (e.g. Robertson 1935). Further the rate of bleeding appears to influence the magnitude of the hemorrhagic hyperglycemia (Mylon et al. 1944; Carey et al. 1972).

In the present investigation the hemorrhagic hyperglycemia response was confirmed. The study showed in addition that the increases of plasma glucose concentration were of such magnitudes as to almost fully account for the concomitantly observed plasma hyperosmolality especially in early stages of hemorrhagic hypotension (Fig. 3 B). This finding by no means excludes the possibility that there are marked changes also of other plasma constituents during hemorrhage. Thus previous studies and the present observations have shown that the concentrations of plasma potassium and various nitrogen compounds increase during hemorrhage (cf. D. Silva 1934; Root et al. 1947; Engel 1952; and Table I). The hyperkalemia apparently is due to a loss of intracellular potassium (cf. Hagberg et al. 1967) and may be an effect of acidosis and/or of cell injury caused by impaired circulation.

TABLE 11 Maximal increase of blood glucose concentration above the control level in different species during various types of hemorrhage

Species	Type of hemorrhage (BV = total blood volume before hemorrhage)	Maximal glucose increase (mg%) ^x	Time after bleeding (min)	Investigator(s)
DOG	Bled 33 % of BV	90 (V)	15	Salto et al 1928
	Bled 70 % of BV	410 (V)	150	Nylon et al 1944
	Bled 75 % of BV	255 (A)	60	Beatty 1945
	Hypotension at 60 mm Hg	480 (A) ^{xx}	90	Brooks et al 1963
	Hypotension at 50 mm Hg	150 (V)	180	Vigas et al 1972
	Hypotension at 40 mm Hg	460 (A)	180	Chien et al 1973
	Hypotension at 30 mm Hg	405 (A)	30	Baue et al 1967
	Hypotension at 30 mm Hg	205 (V)	90	Bauer et al 1969
CAT	Bled 18 % of BV	60 (A)	15	Brooks 1935
	Bled 32 % of BV	440 (A)	20	Robertson 1935
	Hypotension at 50 mm Hg	400 (A) ^{xx}	30	Järhult 1973
RABBIT	Bled 20 % of BV	70 (A)	10	Schenk 1894
	Bled 20 % of BV	70 (V)	125	Epstein and Baehr 1914
	Bled 33 % of BV	230 (A)	30	Andersson 1908
RAT	Bled 50 % of BV	75 (V)	300	Engel et al 1943
SHEEP	Hypotension at 40 mm Hg	125 (A)	75	Halmagyi et al 1966
PIG	Hypotension at 60 mm Hg	180 (V)	60	Carey and Wallack 1970
MONKEY	Hypotension at 50 mm Hg	90 (V)	120	Hiebert et al 1973
BABOON	Bled 45 % of BV	180 (V) ^{xx}	60	Cerchio et al 1971
	Hypotension at 60 mm Hg	100 (V)	30	Moss et al 1970
MAN	Bled 15 % of BV	20 (V)	15	Skillman et al 1971
	(0 mm Hg at admission)	275 (V)	65	Carey et al 1970

^x A = arterial samples V = venous samples

^{xx} Plasma glucose concentration

Much of the potassium seems to emanate from the liver an effect which has been attributed to released adrenaline (Shoemaker et al 1961 Andersen and Shoemaker 1967 Shoemaker 1968) The observed decrease of the plasma sodium concentration in hemorrhage is in accordance with previous findings (Brooks et al 1963 Baue et al 1967) and seems mainly to be the result of an osmotic fluid (water) withdrawal from the intracellular space (see Chapter 6) The chloride bicarbonate and hydrogen ion concentrations can change significantly in hemorrhage and minor alterations of other constituents might also occur (e.g. Wiggers 1950 Engel 1952 Migone 1962)

All these ionic changes in early hemorrhage seem however to virtually cancel each other with regard to the effect on total plasma osmolality In the present experiments the hyperglycemia was thus with few exceptions found to be quite a reliable index of the increase of plasma osmolality

In later stages (>60 min) of hemorrhagic hypotension there was a slow gradual decline of the glucose concentration in face of a maintained hyperosmolality (Fig 3B) This decline might be interpreted as an exhaustion of the liver glycogen stores in fact some authors have reported that animals in hemorrhagic shock may die in a hypoglycemic state (e.g. Engel et al 1943 Strömberg and Hift 1960 Levenson et al 1961 Halmagyi et al 1966) An additional deleterious effect in this phase seems to be marked hyperkalemia In late stages of hemorrhage the present results indicate that osmotically active substances from tissues other than the liver may significantly contribute to the maintained hyperosmolality (cf also Boyd and Mansberger 1968) Such a substance no doubt is lactate which is known to accumulate gradually in plasma during prolonged hemorrhagic hypotension apparently due to impaired oxygen delivery to the tissues (e.g. Beatty 1945 Mastuk and Beatty 1949 Rosenberg and Rush 1968 Rokkanen et al 1971 Schweizer and Howland 1972 Chien et al 1973)

Besides the important hemodynamic effects of the glucose-induced hyper

osmolality per se (see Chapter 6 and 8) the increased plasma glucose concentration during early hemorrhage seems beneficial insofar that it helps to improve the nutritional situation for the tissues. Such an effect would be of special importance for the brain the metabolism of which is highly dependant on an adequate glucose supply. The relative need of glucose in over all cell metabolism might also increase in hemorrhage in view of the fact that the blood concentration of free fatty acids is often reduced after bleeding (e.g. Kováčik et al. 1970; Farago et al. 1971; Spitzer and Spitzer 1972).

Chapter 5

ROLES OF THE SYMPATHO-ADRENAL SYSTEM IN HEMORRHAGIC HYPERGLYCEMIA

(Paper IV)

General considerations

Previous studies have demonstrated that the hemorrhagic hyperglycemia is caused by glucose release from the liver. This was proposed as early as 1894 by Schenk who showed that the hyperglycemia response was abolished by exclusion of the liver from the systemic circulation. Further support for this conclusion was later obtained from direct measurements of the blood glucose concentration in the hepatic vein (e.g. Robertson 1935, Shoemaker et al 1961, Bashour et al 1965, Shoemaker et al 1973). Analyses of the liver glycogen content before and after exsanguination have indicated that the glucose release is mainly due to an increased glycogenolysis (e.g. LePage 1946, Strawitz and Hift 1960).

The stimulus for hepatic glycogenolysis in hemorrhage has commonly been attributed to adrenaline, a hormone known to effectively break down liver glycogen by activation of the adenylyl cyclase system (see Sutherland and Rall 1960). Thus, it has been shown that adrenaline secretion is markedly increased and that the plasma adrenaline level may rise up to 90 times the control value during hemorrhage (e.g. Bedford 1917, Saito et al 1928, Watts 1956, Manger et al 1957, Walker et al 1959, Walton et al 1959, Rosenberg et al 1961, Hall and Hodge 1971, Carey et al 1972). The concept of a dominant role of adrenaline in the hyperglycemia response has been strengthened by the finding that adrenalectomy can cause a decrease or abolition of the hemorrhagic hyperglycemia (e.g. Engel et al 1943, Halmagyi et al 1967, McCormick et al 1969), an effect present also after substitution of glucocorticoids (Hiebert et al 1973).

During the course of the present work we observed however that a hemorrhagic hyperglycemia was still present after bilateral removal of the adrenal glands suggesting a multifactorial control of the glucose release. Indirect support for this hypothesis was also obtained from some recent studies showing an increased plasma glucose concentration during stimulation of the splanchnic nerves in adrenalectomized animals (Edwards 1972a, Edwards and Silver 1972, Bloom et al 1973b). In the series of experiments to be described an attempt was made to define possible different links of the sympatho-adrenal system involved in the hemorrhagic hyperglycemia response.

Present results

The role of the liver in the hyperglycemia response during hemorrhagic hypotension (50 mm Hg) was first to be established. For this purpose determinations of glucose were made in blood sampled simultaneously from the carotid artery and from two sites in the caval vein i.e. at the entrance of the hepatic vein (hepatic vein) and at the iliac bifurcation. Some samples were also taken from the portal and renal veins. The analyses revealed that the plasma glucose concentration in the hepatic vein exceeded that in the arterial blood which in turn exceeded that in the iliac and portal veins (Paper IV see Fig. 2). The glucose level was higher in samples taken from the renal than from the iliac vein but lower than the level in hepatic venous blood. The conclusion was therefore reached that the increased plasma glucose concentration in the present type of hemorrhage experiments is due to release of this substance from the liver in agreement with findings in other hemorrhagic situations (see ref. above).

An attempt was then made to investigate whether the hemorrhagic hyperglycemia could be attributed to hormone release from the adrenals to a sympathetic nervous influence on the liver and/or to a adrenergically in-

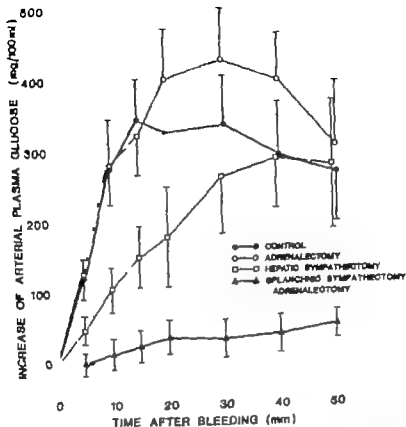
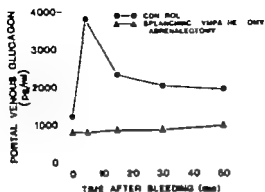


FIG 4 Increases of total plasma glucose concentration above the control level during hemorrhagic hypotension at 50 mm Hg in control animals (n=14), animals with adrenalectomy (n=7), animals with regional hepatic sympathectomy (n=6), and animals with splanchnic sympathectomy combined with adrenalectomy (n=6). Mean values \pm SE are given.

duced release of glucagon from the pancreas. The hyperglycemia response was therefore analysed in animals with an intact sympatho-adrenal system and compared with the response after adrenalectomy, after regional hepatic sympathectomy, and after splanchnic sympathectomy combined with adrenalectomy. In addition, the level of glucagon in the portal venous blood was determined.

Fig. 4 (reproduced from paper IV) shows that the arterial plasma glucose

A



B

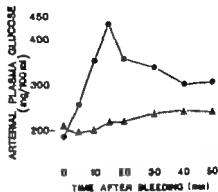


FIG 5 Mean values for portal venous glucagon concentration (panel A) and for arterial plasma glucose concentration (panel B) during hemorrhagic hypotension at 50 mm Hg in 2 cats with an intact sympatho adrenal system and in 2 cats with splanchnic sympathectomy+adrenalectomy

concentration rose rapidly during hemorrhagic hypotension at 50 mm Hg both in the cats with an intact sympatho adrenal system and in the adrenalectomized animals. When the hepatic nerves were sectioned the hyperglycemic response was depressed but only in the early phases of hypotension (<30 min). However when the splanchnic nerves were cut bilaterally in adrenalectomized cats the response virtually vanished.

The level of immuno reactive glucagon in portal venous plasma was determined in 2 cats with an intact sympatho-adrenal system and in 2 cats after adrenalectomy and bilateral splanchnic denervation. In the intact animals hemorrhage caused an almost instantaneous rise of portal venous glucagon concentration whereas no such increase was observed after bilateral splanchnic denervation in adrenalectomized cats (Fig 5 A reproduced from paper IV). The concomitant arterial glucose concentrations are depicted in Fig 5 B showing marked differences between the two groups of animal.

Comments

The present results confirm the opinion that the hemorrhagic hyperglycemia is mainly due to glucose release from the liver. Taken together with previous reports they also indicate that this hepatic glucose release is mediated by at least three different mechanisms: a via a direct sympathetic nerve influence on the liver; b via an adrenergic release of glucagon from the pancreas; and c via a release of catecholamines from the adrenal glands. The study further suggests that these mechanisms quite effectively can compensate for each other: thus after removal of one link a marked hemorrhagic hyperglycemia response was still present. The hyperglycemia as will be discussed below cannot be attributed to a decreased insulin secretion.

Although as mentioned above most previous investigators have suggested adrenaline to be the sole cause of the glucose release in bleeding, there are some earlier studies which support the proposed multifactorial hypothesis. Thus Mylon et al (1944) reported that the hyperglycemia evoked by infusion of large doses of adrenaline into non bled animals was "mild" compared to that seen in hemorrhage, and Carey et al (1972) arrived at the same conclusion. The splanchnic nerve stimulation experiments performed by Edwards group (see above) can also be taken as indirect evidence for the multifactorial hypothesis.

Some details concerning the different links of the sympatho-adrenal system in the hyperglycemia response will be considered.

Sympathetic nerve influence on the liver In the present hemorrhage experiments the glucose level rose by 400 mg% in adrenalectomized animals and the response was depressed after sectioning of the hepatic nerves. These data suggest a direct adrenergic nervous influence on the hepatic glucose release in bleeding, an opinion indirectly corroborated by the following previous observations. Strong excitation of the hepatic nerves (20 Hz) in

adrenalectomized cats leads to an increase of the arterial glucose level by about 200 mg% (Edwards 1972b). Stimulation of the hepatic nerves causes an abrupt rise in the activity of several enzymes involved in liver glycogenolysis (Shimazu and Amakawa 1968a, b). Further recent histochemical studies reveal a rich direct adrenergic innervation of the liver parenchymal cells at least in humans (Hobin et al. unpublished).

Adrenergic glucagon release. The fact that the concentration of portal venous glucagon rapidly increased after bleeding in intact animals but remained unchanged in adrenalectomized animals with severed splanchnic nerves, strongly indicates that glucagon was released via the sympatho-adrenal system. An analogous conclusion was made by Bloom et al. (1973a) who found that glucagon could be released in the monkey in response to other types of stress. Further direct excitation of the sympathetic nerves to the pancreas in cats and calves has been shown to cause a rapid glucagon liberation (Esterhuizen and Howell 1970; Bloom et al. 1973b). In the latter study a close co-ordination in time was found to exist between the increase in glucagon and the subsequent increment of the arterial plasma glucose concentration. The magnitude of this plasma glucose rise amounted to about 100 mg% when the sympathetic nerves were strongly activated. In the present hemorrhage experiments a clearcut glucose increase occurred during the peak glucagon level but the maximum glucose rise was obtained later, apparently due to additional influence from the other control systems. The data taken together indicate that adrenergically induced release of glucagon is involved in the hemorrhagic hyperglycemia response although the quantitative role of this mechanism might be relatively subordinate in comparison to the others. Some observations suggest that glucagon is released also in the late stages of hemorrhage but then as a result of the concomitant hypoglycemia (Halmagyi et al. 1969).

Catecholamine release from the adrenals. As stated above, numerous in

vestigations have demonstrated convincingly that adrenaline and noradrenaline are released from the adrenals in large amounts during hemorrhage contributing to the hyperglycemia response. A detailed study of this mechanism was therefore not considered essential for the present analysis. Our results of a well maintained hyperglycemia after adrenalectomy might at first sight seem to contradict this concept but in all probability can be explained by an effective compensation via the other regulatory mechanisms. A significant contribution of the adrenal medullary hormones to the glucose rise in our experiments in fact seemed established by the results obtained in animals with cut hepatic nerves. Under these circumstances hemorrhage evoked an arterial hyperglycemia of about 300 mg% above control but according to the findings of Bloom et al (1973b) such large a glucose increase cannot possibly be attributed to the glucagon mechanism alone.

Mention should be made here that the hemorrhagic hyperglycemia (and hyperosmolality) response in animals with an intact sympatho-adrenal system was almost totally abolished after α -adrenergic blockade with phenoxybenzamine (5-10 mg/kg) but not after β adrenergic blockade with propranolol (1 mg/kg). Halmagyi et al (1968) made quite similar observations in the sheep. propranolol pretreatment did not change the hemorrhagic hyperglycemia response whereas phenoxybenzamine (1 mg/kg) depressed the response. These findings suggest that the liver glucose release during hemorrhage at least in the cat and the sheep mainly is mediated via α -adrenoceptors (cf Himmels Hagen 1967 Hornbrook 1970).

The present study thus indicates that there are three (main) links of the sympatho-adrenal system which together contribute to the elicitation of the hemorrhagic hyperglycemia response. This does not rule out the possibility that other control mechanisms can be involved as well for instance via release of growth hormone (Meyer and Knobil 1967 Carey et al 1971 Skillman et al 1971) and/or glucocorticoids (e.g. Sayers et al 1945).

Johnson et al 1971 Cryer and Gann 1974) Since in the present experiments the hyperglycemia was virtually abolished when the splanchnic nerves were cut in adrenalectomized cats the effect of the mentioned hormones on the hyperglycemic response must have been quite small in this particular type of hemorrhage

The hyperglycemia response does however not seem to depend upon a hypothetical decrease of the insulin secretion to judge from previous studies Thus the insulin level is reported to be somewhat increased during bleeding in dogs (e.g. McCormick et al 1967 Vigas et al 1972) and virtually unchanged in primates including man (e.g. Carey et al 1970 Moss et al 1970 Cerchio et al 1971 Hiebert et al 1973) The lack of insulin release in response to hemorrhagic hyperglycemia has been attributed to an inhibitory effect of the sympatho-adrenal system (e.g. Hiebert et al 1973 cf. also Malaisse et al 1967 Porte et al 1967 Bloom et al 1973b)

It is quite likely that the hemorrhagic hyperglycemia (and hyperosmolality) response induced via the different links of the sympatho-adrenal system is reflexly controlled by cardiovascular receptors The nature of such a receptor mechanism remains to be elucidated In some preliminary experiments we observed however that the hyperglycemia and hyperosmolality responses were more closely correlated to the induced decrease of arterial blood pressure than to the magnitude of the shed blood volume This finding might be taken to indicate that the response is governed mainly by the arterial mechanoreceptors

OSMOTIC FLUID TRANSFER FROM TISSUES TO BLOOD
DURING HEMORRHAGIC HYPOTENSION
(Paper I III)

General considerations

It was suggested long ago that extravascular fluid enters the circulatory system after a blood loss. This hypothesis was based on observations showing that the concentration of hemoglobin and/or red blood cells was lowered after hemorrhage (e.g. Vierordt 1854, Bierfreund 1891, Koeppé 1895), a hemodilution which seemed to occur mainly in the initial posthemorrhagic period (Boycott and Douglas 1909, Hirota 1928). The phenomenon of a partial plasma volume restoration after bleeding has been confirmed and quantitatively defined in more detail by later studies using different techniques for plasma volume determination. These investigations on animals have revealed that the main fluid replacement occurs within the first hour after bleeding (for ref. see Chien 1967, see also Boyd and Mansberger 1968, Carey 1973, Chien et al. 1973) and similar conclusions have been reached also from experiments in humans (e.g. Ebert et al. 1941, Kaufmann and Möller 1958, Skillman et al. 1967 a).

The mechanism for this hemodynamically important transcapillary absorption of extravascular fluid after hemorrhage was for a long time attributed solely to a passive fall of capillary hydrostatic pressure, secondary to the decline of arterial and venous blood pressure. However, some indications that the sympathetic nervous system might be involved in the process were obtained by Chien (1958). He found that sympathectomy clearly diminished the hemodilution after hemorrhage and that an absorption of extravascular fluid to the blood stream occurred also after bleedings too small to cause significant changes in arterial or venous pressure.

Direct experimental evidence for a net transcapillary absorption of extravascular fluid in response to activation of the sympathetic vasoconstrictor nerves was first presented by Mellander (1960) on a skeletal muscle and skin region in the cat. He also showed that the phenomenon was due to a resetting of the pre-/postcapillary resistance ratio in turn causing a decrease of the capillary hydrostatic pressure. Similar effects of sympathetic nerve stimulation on net transcapillary fluid exchange were observed during regional and hemorrhagic hypotension; the rate of absorption however decreased with time due to impaired reactivity of the precapillary resistance vessels (Lewis and Mellander 1962; Mellander and Lewis 1963). The cardiovascular receptor control of the sympathetically mediated net capillary fluid transfer was investigated in detail by Öberg (1964). Small to moderate hemorrhage of 5-15 min duration consistently caused an augmented vasoconstrictor fibre discharge which led to mobilization of extravascular fluid to the blood stream. Such an autotransfusion was present in skeletal muscle and skin but did not occur in the small intestine. The effect was quantitatively most pronounced in muscle tissue and was clearly detectable even after minor blood losses. Öberg also demonstrated that this reflex fluid redistribution was governed by arterial mechano- and chemoreceptors and later studies indicate that the low pressure receptors might be involved as well (e.g. Öberg and White 1970). Similar studies during more prolonged periods of moderate hemorrhage showed that a fluid absorption from skeletal muscle occurred during the first 25 min and at a maximum rate of $0.1 \text{ ml/min} \times 100 \text{ g tissue}$ after 5 min (Lundgren et al 1964). These results have later been confirmed in essential parts by Haddy's group (e.g. Haddy et al 1965; Schwinghammer et al 1970; Grega et al 1971). Reflexly induced mobilization of extravascular fluid from muscle tissue has also been demonstrated in humans after a 10 % decrease of the circulating blood volume (Mellander and Öberg 1967).

Capillary fluid transfer from the extravascular to the intravascular compartment can be accomplished by mechanisms other than those implicit in the Starling concept. Hyperosmolality of non protein origin has thus been shown to cause a pronounced transcapillary fluid redistribution during heavy exercise both in animals and man (Lundvall 1972, Lundvall et al. 1972). Since in early hemorrhage the tissues are perfused with blood that is hyperosmolar compared to the extravascular fluid (Chapter 3) a prerequisite exists for an analogous osmotic transcapillary fluid absorption. Experiments were therefore designed to reveal such possible fluid transfer in different tissues during bleeding. The analysis indicated that this mechanism indeed is present and participates in the plasma volume control during hemorrhagic hypotension.

Present results

To be able to reveal possible osmotic capillary fluid transfer in hemorrhage the influences of sympatho-adrenal reflex mechanisms and of alterations of arterial and venous pressure on capillary fluid exchange must be eliminated. In the experiments designed for such an analysis reflex effects were excluded by surgical and pharmacological sympathectomy and regional venous outflow pressure was kept constant. The influence of the arterial pressure fall per se on the exchange process will be considered separately (Chapter 7). Observations of the capillary fluid transfer under these conditions were made during hemorrhagic hypotension in three different tissues, viz. skeletal muscle, skin and intestine.

Skeletal muscle Fig. 6 (reproduced from paper II) shows the collected data for the arterio-venous osmolar difference, net transcapillary fluid movement and regional blood flow in the sympathectomized and α blocked cat skeletal muscle region during 90 min of hemorrhagic hypotension at 50 mm Hg. For comparison corresponding data from an intact muscle region are shown

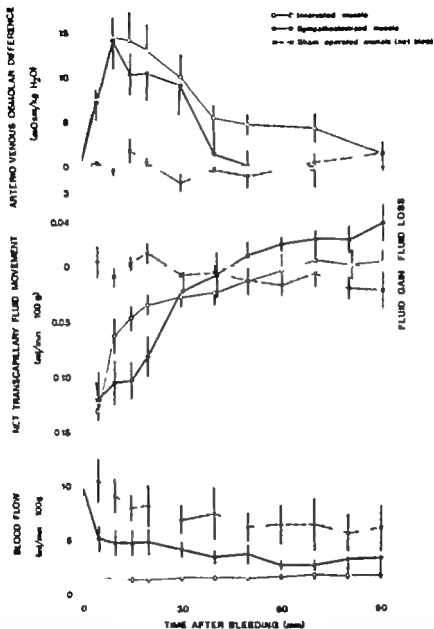


FIG 6 Antero-venous osmolar difference, net transcapillary fluid movement and regional blood flow in an innervated (n 13) and a sympathectomized (14) skeletal muscle region of cats subjected to hemorrhagic hypotension (50 mm Hg) and in sham operated cats (n 4). Data are expressed as mean values \pm SE.

as well as observations from 4 sham operated animals

In the sympathectomized muscle region (Fig 6 solid lines) a net trans capillary fluid absorption occurred as long as the arterio-venous osmolar difference was positive i.e. for about 40 min. The fluid absorption was most pronounced during the initial 20 min of hypotension and the close co ordination in time with the positive arterio venous osmolar difference (reflecting the transcapillary osmolar gradient) indicates that the absorption was due to osmosis (for detailed discussion see below). In the late stages of hemorrhagic hypotension (> 50 min), a moderate transcapillary fluid filtration was observed. The regional blood flow (lower panel) decreased roughly in proportion to the lowered perfusion pressure. If anything, vascular resistance was somewhat reduced in the initial phase (during absorption) and slightly increased in the late phase of hypotension (during filtration).

The net transcapillary fluid absorption in the intact skeletal muscle region during hemorrhagic hypotension at 50 mm Hg (Fig 6 dotted lines) occurred at a somewhat slower rate in certain periods (10-20 min) than in the sympathectomized region despite the fact that the arterio-venous osmolar difference (upper panel) was quite similar in the two types of experiment. This was a somewhat surprising result since it was expected that the reflex mechanism for fluid absorption operating in the intact region would add to the described osmotic fluid absorption. Recent studies by Lundvall (1972) indicate however that the osmotic fluid flux in skeletal muscle is directly dependent both on the arterio venous osmolar gradient and on the magnitude of the regional blood flow. Thus, for a given arterio venous osmolar gradient, an increase of the regional blood flow by 100 per cent was shown to lead to a corresponding increase of the osmotic fluid transfer. This finding is relevant for the present analysis, since sympathectomy clearly augmented the regional blood flow (Fig 6 lower panel). Hence the data for osmotic fluid absorption (solid line) might be an overestimation of what actually occurs in the intact muscle.

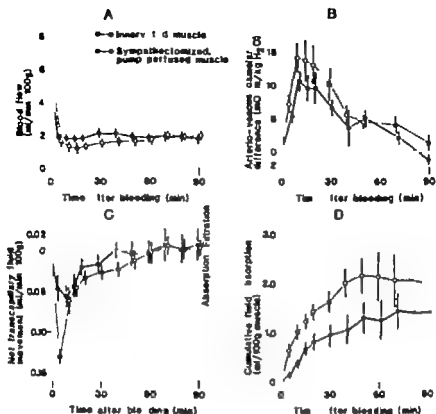


FIG. 7 Comparative data in hemorrhage for arterio-venous osmolar difference (panel B) net transcapillary fluid movement (panel C) and cumulative fluid absorption from the extravascular space (panel D) in the innervated (n 13) and sympathectomized (n 9) muscle region when blood flow in the latter region was mechanically adjusted to about the same level as occurred in the innervated one (panel A). Data for innervated muscle (panel A-C) same as in Fig. 6. Mean values \pm S.E. given.

This problem was approached in some experiments (Fig. 7 reproduced from paper 11) in which the transcapillary fluid absorption during hemorrhage was determined in the sympathectomized muscle when regional blood flow was adjusted by a perfusion pump to the same level as in the intact region (panel A). Since the arterio-venous osmolar difference in such a pump-perfused sympathectomized region was similar to that in the intact muscle region (panel B) the observed net transcapillary fluid transfer in the former was

considered to represent in approximate terms the true osmotic absorption of extravascular fluid in skeletal muscle during hemorrhagic hypotension of this type. The net fluid absorption in the sympathectomized region was clearly diminished by the blood flow reduction (compare Fig. 6 and Fig. 7 C) and was now smaller than in the intact muscle (Fig. 7 C). The analysis performed in Chapter 7 shows that this fluid absorption cannot be attributed to the low arterial inflow pressure per se (30-40 mm Hg) prevailing in the sympathectomized pump perfused tissue.

The cumulative transcapillary fluid absorption in skeletal muscle during hemorrhagic hypotension is depicted in Fig. 7 D in which the dotted curve represents total absorption and the solid curve the fraction caused by osmosis alone (derived from the absorption data in the sympathectomized pump perfused region). After 10 min of hypotension the total amount of absorbed extravascular fluid was 1.0 ml/100 g muscle (35 % osmotic) after 20 min 1.4 ml/100 g (= 55 % osmotic) and after 60 min 2.1 ml/100 g (60 % osmotic). It might be concluded that the adrenergic reflex and the osmotic mechanisms both are important for the plasma volume restoration after hemorrhage and that the reflex mechanism at least in the present type of bleeding is dominant in the early (<20 min) stage of hypotension.

In an attempt to further support the concept of an osmotic transcapillary fluid absorption in hemorrhage the following type of experiment was performed. The osmolality of the blood was increased in non bled adrenalectomized animals (n 5) by slow intravenous infusions of hypertonic glucose solution so as to create an arterial hyperosmolality of similar nature and magnitude to that in the hemorrhage experiments. The blood flow in the sympathectomized muscle region was mechanically adjusted to the same average levels as in the bleeding experiments (cf. Fig. 6 and 7). The rates of fluid absorption resulting from such infusions were found to agree closely with the absorption rates depicted by the solid lines in Fig. 6 and 7 C, espe-

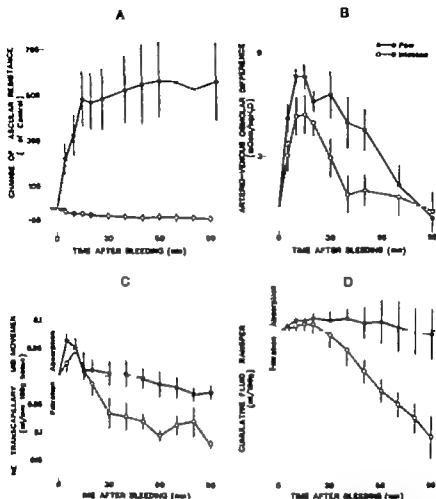


FIG 8 Circulatory events in the sympathectomized α blocked paw (—○— 8 expts) and small intestine (---○--- 8 expts) during hemorrhagic hypotension at 50 mm Hg in the cat. Panel A shows the change of arterial resistance; panel B the arterio-venous osmolar difference; panel C the net transcapillary fluid movement; and panel D the cumulative capillary fluid transfer. Mean values \pm S.E. are given.

cially in the early stage of bleeding.

Skin The changes of plasma osmolality in arterial and cutaneous venous blood revealed the existence of a positive transcapillary osmolar gradient in the sympathectomized and α blocked paw during the first hour of hemorrhagic hypotension at 50 mm Hg (Fig 8 B reproduced from paper III). The

gradient was somewhat lower than in the skeletal muscle due to a less pronounced arterial hyperosmolality in these particular experiments. Observations of net transcapillary fluid movement showed that a moderate absorption of extravascular fluid occurred in the initial period of hypotension (Fig 8 C) when the arterio-venous osmolar difference was most pronounced. Late in hemorrhage a slight fluid filtration was present. The cumulative fluid transfer in the paw during hemorrhagic hypotension is depicted in Fig 8 D. After 40 min a total of 0.8 ml of extravascular fluid per 100 g tissue had been transferred to the circulatory system but this fluid volume was returned to the tissue after 80 min of hypotension due to gradually developing filtration. The vascular resistance in the sympathectomized paw increased markedly during the hypotension (Fig 8 A) most likely due to passive factors as evidenced by regional hypotension experiments (see Chapter 7). It was concluded that the fluid absorption in early hemorrhage was due to osmosis, an opinion supported by hypertonic infusion experiments similar to those described for skeletal muscle.

Intestine Hemorrhagic hypotension at 50 mm Hg evoked only a moderate increase of the arterio-venous osmolar difference in the small intestine (Fig 8 B) compared to that in skeletal muscle; this difference was mainly due to a more rapid increase of the venous osmolality in the intestine. The net transcapillary absorption in the α -blocked intestine was a transient phenomenon (Fig 8 C) and the most prominent observation in fact was a marked fluid filtration in late stages of hypotension. This filtration apparently was due to increased capillary hydrostatic pressure related to the gradual decline of vascular resistance (Fig 8 A). As can be seen from panel D, the cumulative fluid absorption from the intestine was quite small and the main effect was a net loss of plasma fluid amounting to some 5 ml/100 g tissue after 90 min. From these observations the conclusion was reached that the hemorrhagic hyperosmolality caused only a slight transient

fluid absorption from the intestine

Comments

Data on net transcapillary fluid movement in skeletal muscle skin and intestine were derived from plethysmographically recorded changes of tissue volume during constant arterial inflow pressure and venous outflow pressure. Lymph drainage from the regions was effectively prevented by careful cauterization and ligation. Under these conditions a change of tissue volume can represent two events viz a net transcapillary fluid movement and/or an altered regional blood volume (capacitance response). Critical analyses of volume changes recorded with this technique (Mellander 1960 Öberg 1964 1967) have shown that net transcapillary fluid movements can be readily distinguished from capacitance responses an interpretation which has been validated by concomitant use of the ^{51}Cr -method for separate determination of changes of regional blood volume (e.g. Åblad and Mellander 1963 Kjellmer 1965). These analyses have further shown that the capacitance responses almost invariably are closely co-ordinated in time with reactions within the resistance vessels.

The main interest in the present study was focused on net transcapillary fluid absorption and such an effect was found to occur within the first 40 min of hemorrhagic hypotension. The observations of fluid absorption were made after the arterial pressure had reached a steady level of 50 mm Hg (which was accomplished in less than 3 min after the start of the bleeding) and in a state of reasonably stable tone of the resistance and hence of the capacitance vessels. In some individual experiments abrupt minor changes of vascular resistance did occur but then the concomitant capacitance effects were of course disregarded in the analysis of the transcapillary fluid movement. It is therefore concluded that the present approach permitted quite an accurate quantitative evaluation of the transcapillary fluid

absorption in the early stage of bleeding. In the later stages where fluid filtration appeared the analysis might be somewhat less accurate since in this period more gradual minor changes of vascular resistance (and perhaps therefore also of vascular capacitance) were present. The inherent error seems to be small however and possibly cannot alter the main conclusions drawn.

The transcapillary fluid absorption process is of hemodynamic importance for the replacement of plasma volume in hemorrhage. Such fluid absorption occurs in skeletal muscle, skin and intestine but is of major quantitative significance only in muscle due to its very large tissue mass. The main emphasis was therefore placed on the events in this tissue and the mechanisms for the fluid absorption will be considered first.

The study revealed that fluid was mobilized from the extravascular space of skeletal muscle both when the sympatho-adrenal system was intact and after regional α adrenergic blockade and sympathectomy. Earlier studies (see above) have clearly shown that an adrenergic resetting of the pre/postcapillary resistance ratio occurs in hemorrhage leading to decreased capillary hydrostatic pressure and hence to fluid absorption in the intact muscle. It follows that in the α blocked region some other mechanism(s) must have been responsible for the observed fluid absorption and we propose that it is due to osmosis caused by agents other than the plasma proteins. This hypothesis is based on the following findings: a The hemorrhagic hypotension was associated with a pronounced increase of arterial plasma osmolality (Chapter 3); b This hyperosmolality was mainly due to release of glucose from the liver and hence it primarily developed in the blood stream (Chapter 4 and 5); c In the period of fluid absorption clearcut arterio-venous osmolar difference existed in the tissue in turn implying the presence of a concomitant transcapillary osmolar gradient; d The magnitude and time course of the transcapillary fluid absorption were

are closely related to those of the arterio-venous osmolar difference σ . Graded arterial hyperosmolality produced by slow i.v. infusions of glucose in non bled animals evoked fluid absorptions in the α -blocked muscle region of similar magnitudes to those observed in hemorrhage.

It might be argued that the fall of arterial pressure per se during hemorrhage would lower the capillary hydrostatic pressure and cause fluid absorption. This possibility was refuted by the regional hypotension experiments to be described in Chapter 7 which showed that no significant transcapillary fluid absorption was evoked when arterial inflow pressure to the muscle region was mechanically adjusted to 50 mm Hg. The maintenance of capillary pressure in this situation was mainly explained by autoregulatory adjustments of vascular tone.

Several studies have shown that renin, angiotensin and vasopressin can be released during hemorrhage (e.g. Dexter et al 1943, Ginsburg and Heller 1953, Weinstein et al 1960, Skillman et al 1967b, Share 1968, Rocha e Silva and Rosenberg 1969, McNeill et al 1970, Hall and Hodge 1971, Errington and Rocha e Silva 1972). Hypothetically these vasoconstrictors might have influenced vascular tone so as to cause transcapillary fluid absorption. However, direct studies of capillary fluid exchange in skeletal muscle during angiotensin infusion have shown that this agent does not cause any significant fluid transfer (Folkow et al 1961, Järhult 1971) and this seems to be true also with regard to vasopressin (Diana et al 1967). Further, there were no signs of significant release of such vasoconstrictor agents in the present hypotension experiments since vascular resistance in the α -blocked muscle and intestine in fact was decreased during the period of fluid absorption (Fig. 6 and 8).

We therefore conclude that the transcapillary absorption of extravascular fluid from muscle tissue to the blood stream during hemorrhagic hypotension at 50 mm Hg to a significant extent is caused by an osmotic process.

mainly related to the plasma hyperglycemia. The experiments also indicate that the same mechanism is operating in skin and intestine although the osmotic fluid absorption in these tissues is quantitatively less pronounced than in skeletal muscle.

The question then arises from which tissue compartment the absorbed extravascular fluid emanates. The released glucose molecules responsible for the hemorrhagic hyperosmolality will primarily be distributed in the blood stream but secondarily enter the interstitium and increase its osmolality as well. Such an interstitial hyperosmolality must lead to significant osmotic fluid (water) withdrawal from the intracellular space since the reflection coefficient of the cell membranes for glucose can be considered to be close to unity and since the glucose transport into most cells is a slow process.

Experimental support for such a fluid redistribution was obtained from the observed decrease of plasma sodium concentration after bleeding (Chapter 4) indicating a dilution and a relative expansion of the entire extracellular space. This opinion is also in agreement with some direct studies of the extracellular fluid volume in early hemorrhage (e.g. Appelgren 1972). In fact, the osmolar control system in hemorrhage might therefore basically be aimed at a restoration of the extracellular fluid volume but due to the time sequence of the hyperosmolar events it will also institute a fluid redistribution within this space from the interstitial into the intravascular compartment.

Such an osmotic absorption process can be expected to be more effective across capillaries with continuous than with fenestrated endothelium due to the lower solute permeability in the former type (cf Landis and Pappenheimer 1963). The experimental observations also demonstrated such a difference with a more pronounced osmotic absorption in skeletal muscle than in intestine. Even in muscle capillaries however the reflection coefficient

for glucose is far below unity (perhaps in the range of 0.2-0.3 cf Perl 1971) but evidently high enough to create opportunities for glucose-osmotic net fluid movement. Yet the effective driving force for osmotic fluid movement of course is much smaller than what would be predicted from van t Hoff's law. A rough estimate of this driving force (mm Hg) was obtained by dividing the observed rate of osmotic fluid absorption by the capillary filtration coefficient. Such calculations indicated that the mean osmotic driving force during hemorrhage never exceeded 7 mm Hg in the α blocked skeletal muscle. The magnitude of the true transcapillary osmolar gradient cannot be exactly defined in these experiments but for a given average capillary flow velocity it is related to the arterio-venous osmolar difference (for details see Lundvall 1972). At the relatively low flow states prevailing in the muscle during hemorrhage the venous osmolality would be quite representative for interstitial osmolality but the mean transcapillary osmolar gradient of course is considerably smaller than the arterio-venous osmolar difference. Thus if the reflection coefficient is assumed to be 0.25 and the transcapillary glucose-osmotic effective driving force is 7 mm Hg (see above) the value for the mean transcapillary osmolar gradient can be estimated to be about 2 mOsm/kg H_2O .

The present study has shown that hemorrhagic hypotension at 50 mm Hg is associated with considerable transcapillary absorption of extravascular fluid to the blood stream, an event most pronounced in skeletal muscle. In the intact organism this fluid absorption is accomplished by two mechanisms i.e. by the discussed osmolar control and by a reflex adrenergic control (cf Öberg 1964) which lowers capillary hydrostatic pressure via a resetting of the pre/postcapillary resistance ratio. Quantitative data for these two processes in cat muscle tissue were given in Fig. 7 D.

Some aspects of the hemodynamic significance of the hemorrhagic fluid absorption will be considered below and might be most readily appreciated

making an extrapolation of the present data from the cat to man. Such an extrapolation seems to be justified for the following reasons. Reflex fluid absorption from muscle and skin has been demonstrated to occur in the human being after reduction of the circulating blood volume (Mellander and Öberg 1967) and at similar rates to those in the cat, taking the induced hypovolemia into account. Further, severe hemorrhage in man is known to evoke an arterial hyperosmolality and hyperglycemia of similar magnitudes to those reported here for the cat (Boyd and Mansberger 1968, Boyd et al. 1970, Gray et al. 1970). From the data given in Fig. 7 D, it may then be estimated that hemorrhagic hypotension at a level of 50 mm Hg would cause a total reflex fluid absorption of about 250 ml and a total osmotic fluid absorption of about 400 ml from all human skeletal muscles (30 kg) within the first hour of bleeding. The entire fluid absorption from the human muscles accomplished by both mechanisms would thus comprise some 650 ml within this relatively short period of time, a figure compatible with data derived from determinations of the hemodilution after comparable bleeding in man (Kaufmann and Möller 1958).

These considerations indicate that transcapillary fluid absorption in skeletal muscle constitutes a hemodynamically important principle for the plasma volume restoration in hemorrhage and that the osmolar control can be at least as effective as the reflex adrenergic regulation. However, the reflex mechanism is involved already at minor blood losses (cf. Öberg 1964), whereas the osmotic mechanism seems to be put into play first when the bleeding is more pronounced.

Osmotic fluid absorption was shown to occur also in skin and intestine (Fig. 8 D), but these events contribute little to the overall plasma volume regulation. Thus, extrapolation of the data to the whole skin and intestinal tissues in man (each about 2 kg) indicates that the total osmotic fluid withdrawal from skin would comprise only some 20 ml and from intestine some

10 ml in severe hemorrhage

The absorbed extravascular fluid is protein-poor and leads to a decrease of the plasma colloid-osmotic pressure. Yet the absorbed fluid volume is retained within the circulatory system for considerable length of time as evidenced for instance by a maintained hemodilution (e.g. Carey 1973, Chien et al. 1973). There has been no satisfactory explanation of this phenomenon but it appears that it can be understood in view of the described osmolar fluid control. Thus when intracellular water is osmotically absorbed into the interstitial space, a dilution of the colloids in the interstitium of all tissues ensues and hence the interstitial colloid-osmotic pressure decreases. Such an effect would tend to counterbalance the influence of a decreased plasma protein concentration on the Starling transcapillary fluid equilibrium in the body. The efficiency of this mechanism increases if the interstitial protein concentration is high before dilution. Recent studies have indicated that interstitial colloid-osmotic pressure indeed is significant and much higher than previously assumed (for ref. see Aukland 1973 and Lönsmann Poulsen 1974).

The discussed compensatory mechanisms for plasma volume restoration in the present type of bleeding seem to be operating predominantly in the first hour of hemorrhagic hypotension. In later stages, however, a transcapillary fluid loss was observed in all three tissues (Fig. 6 and 8), particularly pronounced in the intestine. Such a fluid filtration has been observed in skeletal muscle and skin in some previous studies (e.g. Lundgren et al. 1964, Hollenberg and Nickerson 1970) and is well established phenomenon in the gut during hemorrhage (e.g. Johnson and Selkurt 1958, Glerert and Pedersen 1967, Cook et al. 1971, Haglund 1973). This fluid loss occurred when the arterio-venous osmolar gradient had vanished and at a time when the neurogenically induced fluid absorption has been reported to be insignificant (Hellander and Lewis 1963). A similar fluid loss was also observed

after prolonged periods of regional hypotension (Chapter 7). It is suggested that this plasma fluid escape was caused mainly by an increased capillary hydrostatic pressure in turn due to passively raised postcapillary resistance. Increased blood viscosity and platelet adhesiveness, cell aggregation, passive collapse of the veins, etc. seem to be factors contributing to this effect (Hardaway et al 1962, Chien 1969, Ljungqvist 1970, Baekstrom et al 1971, Eriksson and Lisander 1972, Ericson and Eriksson 1973). In the intestine, an increase of capillary hydrostatic pressure is compatible also with the observed decline of vascular resistance (Fig. 8 A). Further osmotically active substances (e.g. lactate) seem to be produced in the cells in late stages of bleeding, which also might lead to a fluid withdrawal from the interstitial and secondarily from the intravascular space. By extrapolation of the present data to man, the total transcapillary fluid loss can be calculated to amount about 175 ml after 90 min of hemorrhagic hypotension, of which the major part (≈ 130 ml) occurred in the intestine.

It is likely that during hemorrhage in vivo the transcapillary fluid absorption might be somewhat larger and the fluid filtration somewhat smaller than in the present experiments, since central venous pressure then tends to fall as a consequence of the hypovolemia (cf. Schringhamer et al 1970, Grega et al 1971). Return of extravascular fluid from skeletal muscle and skin to the blood stream via the lymph system apparently must be quite insignificant in view of the exceedingly small lymph flow that has been observed in these tissues under normal conditions at rest (cf. Jacobson and Kjellmer 1964).

Chapter 7

AUTOREGULATION OF CAPILLARY HYDROSTATIC PRESSURE DURING REGIONAL ARTERIAL HYPO- AND HYPERTENSION (Paper III V)

General considerations

Hemorrhage is usually associated with a fall of arterial and central venous pressure. For a long time the transcapillary fluid absorption in hemorrhage was believed to be caused solely by a passive drop of capillary hydrostatic pressure (p_c) secondary to these arterial and venous pressure alterations. To eliminate the effect on p_c of a possible fall of central venous pressure during hemorrhage the present analysis of transcapillary fluid movements was performed under conditions of constant venous outflow pressure in the different regions. Arterial pressure however was reduced from the normal level to 50 mm Hg and it might be argued that this large pressure drop per se could have contributed to the transcapillary fluid absorption described in Chapter 6 via a passive decrease of p_c . However as will be seen from the present analysis such an effect is insignificant.

There are some previous observations which might be interpreted to indicate an approximate maintenance of the Starling fluid equilibrium in skeletal muscle when arterial inflow pressure is decreased (Mellander 1960, Folkow and Öberg 1961, Hanson and Johnson 1962, Lewis and Mellander 1962, Öberg 1964, Thulesius and Johnson 1966, Zweifach 1971) and such a phenomenon seems to be quite well established in the intestine (Johnson and Hanson 1962, Johnson 1968, Zweifach 1971, Haglund and Lundgren 1972, Fronck and Zweifach 1974, Gore 1974). Since skeletal muscle is the main target for the plasma volume control during bleeding there was a need for a more systematic investigation of the influence of arterial alterations per se on the muscle transcapillary fluid exchange and this was done in the present

study in view of the fact that the arterial pressure in vivo varies considerably (Bevan et al 1969) the analysis was limited not only to a pressure level of 50 mm Hg but performed over the whole range from 30 to 170 mm Hg. Some observations were made in the paw as well.

Present results

Skeletal muscle The effects on net transcapillary fluid movement of mechanically induced decreases of arterial inflow pressure were observed in the sympathectomized skeletal muscle with the plethysmographic technique. Similar studies were also made during arterial hypertension in the α -blocked region (see Methodology).

Fig 9 shows the effects of short term (5 min) regional arterial hypotension and hypertension on net transcapillary fluid movement, blood flow and vascular resistance. The data are plotted versus regional perfusion pressure, venous outflow pressure being kept constant at about 5 mm Hg. It can be seen that such short term periods of regional hypotension did not cause any significant transcapillary fluid movement except in the lowest pressure range where in fact a slight fluid filtration was present. Arterial hypertension caused minute filtration. Above a perfusion pressure of 70 mm Hg there was a fairly effective autoregulation of blood flow due to adjustments of regional vascular resistance.

There are no reasons to believe that tissue hydrostatic pressure or the transcapillary colloid osmotic gradient were significantly altered by the adjustments of arterial inflow pressure (for discussion see paper V). From the data on fluid transfer (Fig 9) the conclusion was therefore drawn that p_c remained approximately constant over the entire perfusion pressure range. It follows that the maintenance of p_c under these conditions must be attributed to adjustments of the pre-/postcapillary resistance ratio (r_a/r_v) see Fig 10. For the calculation of r_a/r_v p_c in the control period

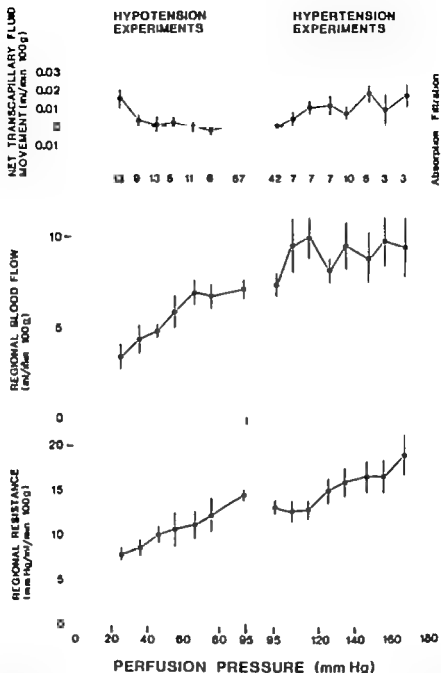


FIG. 9 Cumulated mean data \pm SE on net transcapillary fluid movement and on resistance as responses to lig short term regional arterial hypotension and hypertension in cat skeletal muscle. Indicate number of independent observations.

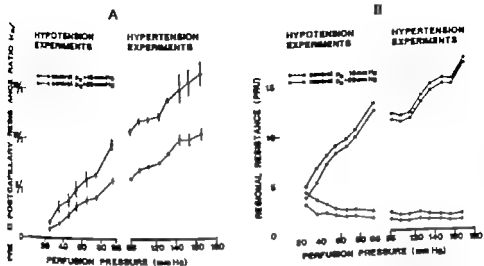


FIG 10 A. Calculated ratios of pre/postcapillary resistance (mean \pm SE) during regional arterial hypo- and hypertension in skeletal muscle for assumed capillary hydrostatic pressures of 15 and 20 mm Hg respectively. The control period with normal perfusion pressure is shown. B. Average precapillary (r_a) and postcapillary (r_v) resistance expressed in absolute figures (mm Hg/ml $\ln \times 100$ g) during regional arterial hypo- and hypertension in muscle.

of normotension must be known (Pappenheimer and Soto Rivera 1948). Recent studies indicate that normal P_c in the skeletal muscle at rest is in the range of 15-20 mm Hg (Diane 1970, Saejo et al 1970, Lundvall 1972, Elias 1974) and both these values were therefore used for the present calculations. It can be seen (Fig 10) that r_a/r_v decreased below the control value during arterial hypotension and increased during hypertension (panel A) and that the alterations except in the lowest pressure range were mainly due to adjustments of precapillary resistance (panel B). The maintenance of P_c during changed arterial inflow pressure was accomplished by active "autoregulatory" adjustments of vascular tone which was clearly demonstrated by comparing the results after abolition of vascular reactivity by papaverine infusions (paper V). In such a passive vascular bed a de-

sure but the effect was mainly caused by a decreased vascular resistance ranging from 10-30 % in the different regions (Fig 13) In relative terms the resistance decrease was most pronounced in skin and skeletal muscle

The fact that the vascular resistance decrease was present in the sympathectomized regions and also after β adrenergic blockade by propranolol (1 mg/kg B wt) indicated that the effect was caused by a direct inhibitory action of the hyperosmolality on the vascular smooth muscle (see below) The blood pressure response to hypertonicity however was depressed by about 40 % by propranolol which suggested that the effect of hyperosmolality on the heart was partly mediated via cardiac β -adrenoceptors (see further below)

Comments

The presents results showed that experimentally induced blood hyperosmolality of similar magnitude to that occurring in severe hemorrhage (Chapter 3) and in heavy exercise (Lundvall et al 1972) can evoke a moderate dilatation of peripheral resistance vessels probably by a direct action on the vascular smooth muscle The mechanisms responsible for the inhibitory action of hyperosmolality on vascular tone have been studied in vitro (Mellander et al 1967 Johansson and Jonsson 1968 Arvill et al 1969 Johansson 1969) These investigations have shown that the smooth muscle relaxation could be ascribed mainly to inhibition of myogenic pacemaker activity in turn due to changes in transmembrane ionic concentration gradients and in membrane permeabilities produced by osmotic reduction of smooth muscle cell volume It cannot be excluded however that part of the resistance decrease was due to rheological phenomena i.e. a lowered blood viscosity caused by absorption of extravascular fluid

The osmotically induced increase of arterial blood pressure in face of

vasodilatation in four hemodynamically important vascular beds indicates an enhancement of cardiac output in turn due to an increased stroke volume since heart rate was unchanged. The increased plasma volume caused by osmotic transcapillary fluid absorption (Chapter 6) quite likely contributed to the observed blood pressure rise and then mainly via an interference with the Frank-Starling mechanism. The cardiac effect at least in part also might be attributed to a direct positive inotropic action of hyperosmolality by a hypothesis which gains support from previous more detailed investigations both in vitro and in vivo (Koch-Weser 1963, Wildenthal et al 1969a, b, Templeton et al 1972, Atkins et al 1973). The attenuated blood pressure response to hyperosmolality after β blockade suggests that the cardiac effect to some extent also was mediated via β adrenoceptors, an opinion corroborated by the studies of Wildenthal et al (1969a). That hyperosmolality can lead to a centrally mediated increase of sympathetic discharge has recently been shown by Mellander and Hillman (1975) and Schad and Sailer (1975).

It may be concluded that hyperosmolality is a factor which can contribute to the overall cardiac adjustments in hemorrhagic hypotension but simultaneously tends to oppose the reflex peripheral vasoconstriction.

The described effects were evoked by what can be considered to be a physiological increase of blood osmolality. The pattern of response to hyperosmolality can be different when drastic rapid increases of blood tonicity are experimentally induced. Under these circumstances a bradycardia and arterial hypotension most commonly develops (e.g. Binet and Stoicescu 1929, Muirhead et al 1947, Read et al 1960) but some authors have reported different neurogenic vascular resistance responses mediated via central or peripheral receptors (Holland et al 1959, Lasser et al 1960, Agarwal et al 1969, Inglesby et al 1972, Raizner et al 1973).

Chapter 9

GENERAL CONCLUSIONS

The aim of the present investigation was to define blood osmolality changes during hemorrhagic hypotension their underlying mechanisms and their importance for circulatory adjustments in hypovolemia. The experiments were performed on anesthetized cats exposed to standardized rapid bleeding to a constant arterial pressure level of 50 mm Hg. The following main conclusions were drawn:

1 Hemorrhage caused a rapid increase of arterial plasma osmolality reaching a level about 20 mOsm/kg H_2O above the control value after 20 min. This plateau was then maintained throughout a hypotension period of 3 hours. The osmolality in peripheral venous blood rose more gradually implying the existence of a positive arterio-venous osmolar difference during the early stage of hypotension.

2 A pronounced hyperglycemia developed during hemorrhage and its magnitude was such as to almost fully account for the blood hyperosmolality during the first hour of hypotension. Electrolyte changes also occurred but in opposite directions (e.g. decreased sodium and increased potassium concentrations) so that the net effect of such alterations on total plasma osmolality was small.

3 The hemorrhagic hyperglycemia was caused by glucose release from the liver in turn mediated via three different reflex mechanisms: by direct sympathetic nervous influence on the liver, by glucagon released via the sympatho-adrenal system and by catecholamines emanating from the adrenal medulla.

4 Quantitative analysis of capillary fluid exchange in muscle revealed a considerable transcapillary absorption of extracellular fluid into the blood stream during the first hour of hemorrhage.

5a fluid transfer occurred not only in the intact muscle region but also after regional α -adrenergic blockade and sympathectomy showing that mechanisms other than the previously known adrenergic reflex resetting of the pre/postcapillary resistance ratio contributed to the fluid absorption

5 This fluid absorption could not be attributed to a passive fall of capillary hydrostatic pressure (p_c) caused by the arterial hypotension per se, since p_c was shown to remain virtually constant at the normal control level when arterial inflow pressure to the muscle region was mechanically varied over the whole range from 30 to 170 mm Hg in non bled animals. This maintenance of p_c was accomplished by local autoregulatory adjustments of precapillary vascular tone causing a resetting of the pre-/postcapillary resistance ratio

6 The fluid withdrawal was not due to a fall of regional venous pressure since this was kept constant in the present experiments. Nor could it be ascribed to the effects of humoral vasoconstrictor agents such as angiotensin or vasopressin

7 The rate and time-course of the transcapillary fluid absorption in the sympathectomized and α blocked muscle region were distinctly related to the observed arterio venous osmolar difference (reflecting the transcapillary osmolar gradient). Absorption at similar rates to those observed in hemorrhage was evoked in the sympathectomized muscle region in non-bled cats in response to hypertonic glucose infusions which raised plasma osmolality to the same levels as in bleeding. The conclusion was reached that the described capillary fluid transfer was an osmotic process caused by the arterial hyperosmolality (hyperglycemia)

8 Osmotic withdrawal of extravascular fluid also occurred in skin and intestine during hemorrhage. This effect especially in the intestine was less pronounced than in skeletal muscle

9 The transcapillary absorption of extravascular fluid from skeletal

muscle is of great hemodynamic importance in hemorrhage insofar that it helps to restore plasma volume. The present data indicated that in the intact organism the adrenergic reflex and the osmotic mechanisms are both important for this plasma volume control - the former mechanism being especially effective in the initial stage of hemorrhagic hypotension. Extrapolation of our data on capillary fluid transfer to man suggests that a total extravascular fluid volume of about 650 ml would be absorbed into the blood stream from all skeletal muscle tissue within the first hour of hemorrhagic hypotension. The osmotic mechanism would be responsible for at least 50 % of this effect.

10 It appears that the described osmolar control system is basically aimed at an overall regulation of extracellular fluid volume in hemorrhage insofar that it causes a glucose-osmotic fluid (water) withdrawal from the intracellular compartment. The consequent dilution of proteins in the interstitial space of the tissues can help to explain why the osmotically and reflexly absorbed protein-poor extravascular fluid from skeletal muscle and skin is retained for considerable length of time within the circulatory system.

11 In later stages of hemorrhage some plasma fluid was lost by filtration into skeletal muscle, skin and intestine - an effect most pronounced in the latter tissue.

12 In addition to the effect on fluid redistribution the hemorrhagic hyperosmolality was found to exert a positive inotropic action on the heart and a moderate dilator effect on the resistance vessels in skeletal muscle, skin, intestine and kidney.

It appears from this and previous studies that the organism with regard to its volume control has several defence mechanisms against hemorrhagic hypovolemia. a Reflex adrenergic constriction of the capacitance vessels

tends to adapt almost instantaneously the size of the intravascular space in relation to the decreased blood volume and this effect is co-ordinated with reflex adjustments of cardiac performance and peripheral resistance

b Reflex adrenergic resetting of the pre /postcapillary resistance ratio leads to withdrawal of interstitial fluid from muscle tissue into the blood stream hence increasing the plasma volume

c The reflexly induced glucose-osmotic mechanism tends to restore extracellular fluid volume by water redistribution from the intracellular space and in addition it increases the plasma volume by net transcapillary fluid movement from skeletal muscle

d By activation of the thirst mechanism and adjustments of renal excretion, the overall fluid balance in the body tends to be restored

e Finally there is a gradual restitution of the red cell volume mainly effected by the erythropoietin mechanism

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 444

INTERACTION BETWEEN CENTRAL NEUROGENIC MECHANISMS
DESIGN

Experimental studies in spontaneously hypertensive rats

by

MARGARETA HALLBÄCK

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 424

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**INTERACTION BETWEEN CENTRAL NEUROGENIC MECHANISMS
AND CHANGES IN CARDIOVASCULAR DESIGN
IN PRIMARY HYPERTENSION**

Experimental studies in spontaneously hypertensive rats

by

MARGARETA HALLBACK

This summary is based on studies reported in the following papers:

- I Cardiovascular responses to acute mental stress in spontaneously hypertensive rats M Hallböck and B Folkow Acta physiol scand 1974 90 684-698
 - II Consequence of social isolation on blood pressure cardiovascular reactivity and design in spontaneously hypertensive rats M Hallböck Acta physiol scand 1975 In press
 - III Background of increased flow resistance and vascular reactivity in spontaneously hypertensive rats B Folkow M Hallböck Y Lundgren and L Weiss Acta physiol scand 1970 80 93 106
 - IV The distensibility of the resistance vessels in spontaneously hypertensive rats (SHR) as compared with normotensive control rats (NCR) M Hallböck Y Lundgren and L Weiss Acta physiol scand 1974 90 57 68
 - V Consequences of myocardial structural adaptation on left ventricular compliance and the Frank-Starling relationship in spontaneously hypertensive rats M Hallböck O Isaksson and E Norell Acta physiol scand 1975 In press
- The papers are referred to in the text by their Roman numerals

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INTRODUCTION

The background of the increased arterial pressure in essential hypertension has been subjected to numerous investigations but no sole or main cause has been revealed so far (cf. Pickering 1968). It rather appears as if this "disease of regulation" (Page and McCubbin 1965) is the result of an altered interaction between the various mechanisms involved in normal blood pressure regulation in such a way as to reset the pressure equilibrium to a higher level in essential hypertension. Although many different opinions exist concerning the etiology of essential hypertension, most investigators nowadays agree on the presence of several predisposing hereditary elements (cf. Mall 1971). It is here of interest that also the most common types of hereditary hypertension in rats developed by selective inbreeding (Smirk and Hall 1958; Okamoto and Aoki 1963) are characterized by a polygenic inheritance. Among these animal models of man's essential hypertension the Okamoto type of spontaneously hypertensive rat (SHR) has been particularly well studied, exhibiting several close parallels to the situation in man with an important involvement of particularly neurohormonal mechanisms (cf. Okamoto 1972; Folkow 1975).

For such reasons SHR were utilized in the present series of studies which are below denoted I-V. The main purpose was to elucidate the relative roles and temporal interactions of functional and structural elements for the initiation and maintenance of the hypertensive state. Study I was designed to explore possible differences between SHR and normotensive control rats (NCR) concerning centrally induced cardiovascular responses to graded alerting stimuli and if a hyperreactivity in this respect could constitute a genetically linked factor predisposing for hypertension in SHR. Study II was aimed to elucidate whether normal environmental influences *per se* in the animal's daily life interact with the mentioned hyperreactivity in such a way as to contribute crucially to the gradual initiation of the hypertensive state.

Although the results suggest that such an interaction of central excitatory influences seems to exert a most important triggering effect it was evident from earlier studies both in man (Folkow 1956; Conway 1963; Siverthsson 1970) and in SHR (cf. Weiss 1974; Lundgren 1974) that the secondary but rapid establishment of structural cardiovascular changes appears to be a prerequisite for the creation of chronic hypertension. Interest was therefore also focused on the extent and type of structural adaptation of the resistance vessels (III+IV) and its importance for the maintenance of an increased flow resistance and pressure in SHR hypertension.

Finally many studies in man indicate that the hemodynamic situa-

tion in primary hypertension may often start as a hyperkinetic circulatory state with an increased cardiac output (for ref see Julius and Schork 1971) to be gradually transferred into a state of normal or even slightly lowered cardiac output but with a raised resistance (e.g. Eich et al 1966). It was therefore explored in SHR whether this shift in hemodynamic balance along with progression of hypertension also might depend on the gradual cardiovascular structural adaptation but then rather on the development of left ventricular hypertrophy.

HEMODYNAMICS OF PRIMARY HYPERTENSION

Early or labile hypertension

Although nomenclature and definitions vary considerably this phase of hypertension is generally defined as the state when arterial pressure values vary around the borderline between normal and abnormal. Therefore the term borderline hypertension is frequently used (cf Julius and Schork 1971). Already in 1939 Wezler and Böger recognized in young patients with hypertension that the increased blood pressure was sometimes accounted for by an increased cardiac output (CO) later confirmed by e.g. Werkö and Lagerlöf (1949), Widimský, Fejfarová and Fejfar (1957), Bello Sevy and Harakal (1965), Sannerstedt (1966), Lund Johansen (1967), Julius and Conway (1968), Frohlich et al (1970). This increased cardiac output observed in 30-50 per cent of patients with labile hypertension (for ref see Julius and Schork 1971) is sometimes ascribed to an increased stroke volume (Widimský, Fejfarová and Fejfar 1957, Finkelstein, Worcel and Agrest 1965, Bello Sevy and Harakal 1965) but more commonly to an increased heart rate (e.g. Sannerstedt 1966, Lund Johansen 1967, Julius and Conway 1968, Frohlich et al 1970) or a combination of both (Eich et al 1966). However the relationship between cardiac output and oxygen consumption seems to be largely normal both during rest and during exercise in labile hypertension (Julius and Conway 1968, Lund Johansen 1967, Sannerstedt 1966). The regulation of cardiac output appears therefore to be essentially normal but starts from a higher baseline level (Sannerstedt, Julius and Conway 1970).

The mechanisms behind the increased cardiac output or hyperkinetic circulatory state present in the resting situation are not fully understood. It has been proposed that a primary volume expansion and thus increased venous return leads to an increased cardiac output and then to a secondary rise of resistance that is assumed to be due to whole body autoregulation (Coleman, Granger and Guyton 1971). Such a train

of events may well occur in case a primary volume increase really is at hand. However subjects with early essential hypertension appear to display a normal or even reduced circulating blood volume (e.g. Bello Savy and Horakal 1965 Julius et al 1971 a) pointing to other types of initiating mechanisms. In fact most subjects with labile hypertension display obvious signs of an increased sympathetic discharge to the heart in association with a decreased vagal activity (Julius Pascual and London 1971). In addition they seem to show a centralization of their reduced blood volume per se (Ellis and Julius 1973) with a consequently increased filling of the heart — a phenomenon which may be indicative of an increased sympathetic activity to the cardiovascular system notably to its venous side (Walsh Hyman and Maronde 1969 Brod et al 1974).

Together these findings strongly indicate an early involvement of an increased central neurogenic drive to the cardiovascular system by its balance suggesting a mild defence reaction which seems to be present in many though by no means in all cases of early essential hypertension (cf Falkow 1975). Peripheral resistance was earlier thought to be largely normal in labile hypertensives. However when related to their raised cardiac output they display elevated total peripheral resistance compared with normotensives with similar resting cardiac output (Julius and Conway 1968). An increased flow resistance in labile hypertensives was also evident during mild exercise (Sannerstedt 1966 Lund-Johansen 1967). Therefore it has been stated that a disturbed balance between cardiac output and resistance is responsible for the pressure elevation rather than the raised cardiac output alone (e.g. Widimský Fejfarová and Fejfar 1957 Julius et al 1971 b).

Established hypertension

Already more than two hundred years ago a well conceived concept of what is now called essential hypertension was described by Samuel Schar-schmidt (cf Backer 1953). However since no technique for indirect blood pressure measurement was available at that time investigators mainly relied upon postmortem examinations of the cardiovascular system. Thus when Bright (1836) described a number of cases with degenerative kidneys — later called chronic Bright's disease — he noted among other things — marked left ventricular hypertrophy. Later investigations (Johnson 1868 Gull and Sutton 1872 Ewald 1877) also revealed an altered morphology of the arteries and arterioles which was even considered to be the primary or essential condition of the chronic Bright's disease. Mahomed (1881) being one of the first to measure arterial pressure clinically demonstrated the presence of an increased pressure in most cases of Bright's disease. He also proposed that the cardiovascular changes

may precede the renal changes which was also considered by e.g. Huchard (1889). Thus the concept that hypertension can precede kidney lesions is much older than the term essential hypertension which was introduced first 1911 by Frank.

Already Bright found an increased resistance to flow in diseased kidneys (cf. Backer 1953) and since then an increased systemic resistance to flow has been considered the main feature in established essential hypertension. Cardiac output is by and large normal although somewhat changed in its distribution with a modestly increased supply to the skeletal muscles at the expense of the renal and splanchnic vascular beds (cf. Fries 1960, Brod et al. 1962, Pickering 1968). According to Poiseuille's law resistance to flow is determined by the radius, length and number of the tubes and the viscosity of the perfusate. There are good reasons to assume that effective blood viscosity is largely normal in established essential hypertension (for ref. see Pickering 1968, Lundgren 1974, Weiss 1974). Concerning vascular dimensions in hypertension an increased length of the resistance vessels has not been convincingly documented (cf. Pickering 1968) and an average reduction of vascular diameter is by far the main determinant of flow resistance particularly since resistance varies inversely to the fourth power of the internal radius. Therefore most studies of the hemodynamics in established hypertension have been concentrated on the background of the reduction in average resistance vessel radius.

Background of reduced resistance vessel radius

It was earlier taken more or less for granted that the raised resistance in established essential hypertension was largely a matter of a sustained increase in vascular smooth muscle activity and the main controversy was how such an assumed increase of smooth muscle activity was induced. Thus it has been suggested to be caused e.g. by increased neurogenic or hormonal excitatory influences, by a raised smooth muscle sensitivity to such extrinsic influences or/and by an increased myogenic tone perhaps resulting from a changed ionic balance across the muscle cell membranes. Little if any attention was paid to the possibility that the structural changes in arteries and arterioles which have been recognized for more than 100 years could be of crucial importance for the raised resistance until this aspect was taken up for critical analysis some 20 years ago (for ref. see Folkow et al. 1973).

Concerning the abovementioned alternatives for increasing smooth muscle activity it seems clear that mechanisms of this nature are likely to play a role as initiating factors and furthermore that they probably differ in nature in the various types of hypertension. For example hormonal influences are clearly of crucial significance in some secondary types of hypertension like renal hypertension, Conn's syndrome, Cushing's

disease and pheochromocytoma (cf Pickering 1968)

The increased resistance may as previously mentioned also be the result of altered ionic composition of the vascular smooth muscles (cf Friedman Friedman and Nakashima 1957 Pickering 1968) or/and reflect a water logging of the vessel wall (Tobian and Blanton 1952). In SHR it has been suggested that an altered ionic transport in vascular smooth muscle of large arteries may account for an increased reactivity to noradrenaline (Jones 1973). However essentially all studies of a g SHR vascular strips reveal a normal or slightly reduced reactivity to noradrenaline (see below). Moreover there is no difference in sodium potassium and water content in SHR arteries in the very early prehypertensive stage suggesting that any electrolyte disturbances which may appear later are rather a consequence than the cause of hypertension (Nagaoka Kikuchi and Aomaki 1970).

Concerning neurogenic effects which will be discussed separately below there are several findings indicating enhanced sympathetic influences both in essential hypertension in man and in SHR. However it is difficult to judge whether such quantitative differences really reflect a tonic increase in sympathetic activity or rather a more transient increase as a result of a raised central reactivity in the hypertensive subjects when exposed to the apprehension and discomfort of investigation. Hence the obtained differences in neurogenic influences between groups might rather reflect differences during experimental conditions that are not necessarily present during truly resting conditions (cf Pickering 1968 Folkow 1975).

A great many studies illustrate how most hypertensive subjects are hyperreactors to a variety of vasoactive substances where predominantly the adrenergic transmitter noradrenaline has been used (for ref see Doyle 1968 Mendlowitz 1973). The background of such a changed vascular reactivity can be complex indeed (cf Johansson 1974) but such findings generally have been taken to reflect either a genetically linked hypersensitivity/reactivity of the smooth muscle cells per se (e.g Doyle and Frazer 1961) or an inherent abnormality of noradrenaline metabolism (e.g Mendlowitz et al 1962 Mendlowitz 1973). However the increased vascular reactivity can also be due to an altered design of the resistance vessels in the direction of an increased wall/lumen ratio as will be dealt with in more detail below. Thus while a vascular hyperreactivity is no doubt at hand when tested as resistance changes (cf Doyle 1968) most studies performed on vascular smooth muscle strips a technique which eliminates the impact of a changed wall/lumen ratio report a largely equal or even a reduced responsiveness to a g noradrenaline in strips from cases with primary hypertension and normotensive controls (e.g Spector et al 1969 Clineschmidt et al 1970 Hallböck Lundgren and Weiss 1971 Horwitz et al 1974). Furthermore in

quantitative studies of the threshold sensitivity of the resistance vessels to noradrenaline which are here more relevant than preparations from large arteries no signs of any altered smooth muscle sensitivity to the adrenergic transmitter have been observed either in human essential hypertension or in SHR (Silverthorn 1970, Finch and Hoessler 1974). However vascular hyperreactivity was clearly observed which suggests an altered design of the resistance vessels.

Hemodynamic consequences of altered design of the resistance vessels

As mentioned media hypertrophy was recognized in hypertensive disease long before its modern classification by Johnson (1868) and Ewald (1877) who even speculated whether the structural arteriolar changes might be crucially involved in the very establishment of the hypertensive state. Since then the presence of an increased wall thickness reducing the wall/lumen ratio of the arterioles in hypertension has been well documented by numerous morphological studies (e.g. Kernohan, Anderson and Keith 1929; Moritz and Old 1937; see also Pickering 1968).

Thus although media hypertrophy has generally been considered to be the main cause of the increased wall/lumen ratio in hypertensive arterial vessels other types of changes have also been suggested such as an increased intramural water content i.e. "water logging" (Tobian and Binion 1952) or a vascular "contracture" (Short and Thomson 1959, Short 1966). Furthermore with morphological techniques the problems of quantitation are great and a number of artifacts has to be considered e.g. differences in smooth muscle activity at the moment of fixation difficulties to define comparable sections of the vascular bed differences in transmural pressure and vascular distensibility etc. Such difficulties were to a great extent avoided by Short (1966) in his careful studies. Suwa and his group have also very elegantly solved most of these difficulties (Furuyama 1962, Suwa and Takahashi 1971). The latter group found a close correlation between blood pressure and degree of media hypertrophy in arteries and precapillary resistance vessels with the exception of their most distal sections which were considered to be protected from any pressure rise by the resistance increase in more proximal sections.

Thus it can be concluded that an increased wall thickness leading to an enhanced wall/lumen ratio in the resistance vessels in hypertension has been well documented with modern morphological techniques. However such methods can hardly compete in accuracy with hemodynamic evaluations of vascular dimensions since with the latter approach the measured variations in flow are amplified to the fourth power of changes in internal vascular radius. Further the transmural distending pressure can be far better defined as can also the level of smooth muscle activity. Moreover with morphological studies only occasional vessels can be sampled for

statistical analysis whereas the hemodynamic approach automatically provides information about the average change in design of all the resistance vessels.

However when evaluating the structurally determined component of the increased resistance in hypertension by the hemodynamic approach it is naturally all-important to first eliminate all extrinsic and intrinsic interferences with smooth muscle activity. First when they are completely relaxed an adequate baseline is provided from which smooth muscle activations can be induced and their effects on dimensions, distensibility, etc. of the resistance vessels can be estimated. In other words in situations of maximal dilatation and standardized transmural pressures the flow resistance reflects the resting length of the circumferentially arranged smooth muscle coat. This level forms the necessary starting point for judging the extent of smooth muscle contraction by the induced increase in resistance. Thus only when comparing the resistance during maximal dilatation with that during normal basal levels of smooth muscle activity in different vascular beds it is possible to see whether this basal vascular tone differs in the various vascular beds (cf Celander and Folkow 1953, Lövling and Mellander 1956).

Concerning hypertension an increased forearm (or hand) resistance to flow has been demonstrated both during rest i.e. at basal vascular tone and when vasodilatation was induced by nerve blockade, indirect heating or by arterial occlusion (Prinzmetal and Wilson 1936, Pickering 1936). However at that time a vascular hypertonus rather than structural changes was considered to cause the raised resistance. The importance of structural vascular changes for maintaining an increased flow resistance in hypertension was brought into focus when Folkow (1956), Folkow, Grimby and Thulesius (1958) and Conway (1963) utilized the principles of analysis outlined above for comparing different systemic circuits and by special procedures ensured a complete smooth muscle relaxation of the forearm resistance vessels. Thus quantitative comparisons of this regional resistance at maximal dilatation and in the resting situation in patients with essential hypertension and in normotensive controls could be performed. These quantitative hemodynamic measurements in the forearm in association with the morphologically well documented media hypertrophy in hypertension illustrate how an increased wall thickness when encroaching upon the vascular lumen raises the resistance even during maximal dilatation. Since e.g. neurogenic activations of the media are initiated from its adventitial layer where the neuroeffector junctions are concentrated (cf Johansson et al 1970) the reduction of the internal radius becomes exaggerated in vessels with a thicker media because of the greater bulk of tissue that is forced towards the lumen upon smooth muscle shortening (Folkow 1956, Folkow, Grimby and Thulesius 1958). These exaggerated resistance increases thus

imply a vascular hyperreactivity that does not necessitate any smooth muscle hyperreactivity or -sensitivity

To summarize in the presence of media hypertrophy an increased resistance can be maintained in hypertension at a normal activity of the smooth muscle per se because first the very baseline for their operation is raised and second a given muscle shortening leads to exaggerated luminal reductions thanks to the changed design. In fact amplified variations in resistance both in the direction of constriction and dilatation will result from the presence of a structural increase in wall/lumen ratio as long as no sclerotizing processes are also involved (for further details concerning theoretical considerations see Lundgren 1974, Weiss 1974 and study III)

THE NEUROGENIC COMPONENT IN PRIMARY HYPERTENSION

From what has been mentioned above it follows that the extent of resistance change in hypertension does not directly reflect the extent of vasoconstrictor fibre activity since the more the wall/lumen ratio of the resistance vessels is structurally increased the more will such a factor amplify the resistance changes. Therefore when comparing the extent of adrenergic fibre activity in hypertensives and normotensives by analyzing the effector response it is necessary to use effectors where possible changes of design will not affect the response like heart rate or venous capacitance response. Even then the presence of e.g. an effector supersensitivity may result in exaggerated response to a given of fibre discharge.

However the reduced venous distensibility observed in patients with essential hypertension in all likelihood reflects an increased sympathetic activity (Walsh, Hyman and Maronde 1969, Brod et al 1974). Such an interpretation is supported by an analysis of the venous compartment in SHR hypertension where there is no evidence of any altered design or true effector supersensitivity (cf Folkow et al 1974). Further analyses of cardiac neurogenic control in early hypertension (e.g. Julius Pascual and London 1971) strongly indicate the presence of an increased sympathetic activity in essential hypertension of man.

Another reasonably good indicator of overall changes in sympathetic activity can be obtained by measurements of the release of the sympathetic transmitter noradrenaline (NA) provided that the amount released per stimulus and its rate and mode of elimination has not been changed. Initially the urinary NA excretion rate was measured since it was found to increase during generalized increases in sympathetic activity as induced by tilting, exercise, mental stress etc (cf Euler 1956) in

many investigations normotensive and hypertensive subjects were compared in this respect but with rather inconsistent results (for ref see Brunjes 1964 De Quattro and Miura 1973). With the development of sensitive methods for measuring catecholamines in blood a more direct reflection of sympathetic activity was available and most results with this technique suggest a moderately increased sympathetic activity in patients with essential hypertension (Engelman Portnoy and Lovenberg 1968 Engelman Portnoy and Sjoerdsma 1970 Louis and Doyle 1971 Louis *et al* 1974). The plasma concentration of dopamine β -hydroxylase (DBH) which converts dopamine to noradrenaline in the nerve endings and is released upon sympathetic activation has also been demonstrated to be higher in hypertensives than in normotensives (Louis *et al* 1974) although the range of variability in resting normotensives may be too large to allow for any reliable quantitation (e.g. Rush *et al* 1974 see also De Quattro and Miura 1973).

Direct recordings of sympathetic fibre activity to the vessels of skeletal muscle and skin have recently been performed in man (Wallin Deltus and Hagbarth 1973) and the general pattern of sympathetic discharge appears to be qualitatively the same in hypertensive and normotensive subjects. Although so far the technique has not allowed any clear-cut quantitative separation between the two groups the findings illustrate like those of Wagner Wackerbauer and Hilger (1968) Korner *et al* (1974) and others the resetting of the arterial baroreceptor reflex in human essential hypertension which was first shown experimentally in dogs and rabbits with renal hypertension (McCubbin Green and Page 1956 Aars 1969). An intact baroreceptor function but reset to a higher pressure level has also been demonstrated in SHR (Nasaka and Okamoto 1970 Nasaka and Wang 1972).

With SHR as an animal model for primary hypertension the involvement of neurogenic mechanisms has been extensively explored using morphological histochemical as well as physiological methods. In general these studies suggest an increased neuro-hormonal rather than a purely neurogenic participation involving not only the sympatho-adrenal system but also the ACTH corticoid and TSH thyroxine systems (cf Okamoto 1969 1972). Furthermore in young anesthetized SHR exposed to surgical interventions an increased heart rate and cardiac output have been demonstrated which seem in part to be due to an increased sympathetic activity to the heart (Pfeffer 1972 Pfeffer *et al* 1974). Very young SHR also seem to display an increased cardiac NA turnover when compared with NCR (Yamori 1974). However most comparative studies on older SHR and normotensive controls suggest if anything a lower NA turnover rate in SHR hearts (Louis *et al* 1969 Okamoto 1972). The advantage with this approach even though indirect and with several pitfalls is that the average degree of sympathetic activity can be

followed during conditions when the animals are largely undisturbed. Other evidence indicating an increased cardiac sympathetic activity in young SHR is the effectiveness of early β -adrenergic blockade treatment preventing blood pressure to increase when administered to young pre-hypertensive animals while the same treatment is relatively inefficient in older SHR (Weiss 1974, Conway 1975).

When combined the above mentioned findings indicate an increased neurogenic influence on the cardiovascular system in both human and SHR hypertension at least in early phases. It is hardly possible to reveal the exact nature of the enhanced autonomic discharge, i.e. whether it is tonic and persistent in nature or mainly episodic and intermittent. However, what has so far been revealed concerning the enhanced neurohormonal activity in early SHR hypertension and in early hypertension in man with signs of e.g. an increased sympathetic and decreased parasympathetic activity to the heart (Julius Pascual and London 1971, Pfeffer 1972) closely resembles the pattern normally elicited via corticohypothalamic autonomic centres during arousal, i.e. the defence reaction.

The defence reaction

Experimental studies of mild emotional stress in man (e.g. forced mental arithmetics) have shown an increased cardiac output, increased forearm blood flow associated with cutaneous and renal vasoconstriction resulting in an increased arterial pressure (Brod 1963). This hemodynamic pattern corresponds in virtually all details to the neurohormonal adjustments evoked in animals during the defence reaction (Eliasson et al. 1951, Abrahams, Hilton and Zbrozyna 1960). Like several other hypothalamic response patterns this reaction, preparing for flight or flight, involves both autonomic and hormonal adjustments suited to meet the particular demands that the situation may call for, and these adjustments often precede the ultimate motor response. They include a central vagal inhibition to the heart in association with an increased sympathetic activity to the heart, venous side and most vascular circuits, except to that of the skeletal muscles where instead sympathetic cholinergic vasodilator fibres and/or adrenaline secretion contribute to a powerful vasodilatation (cf. Folkow and Neil 1971). The balance between the vasodilatation in skeletal muscles and the vasoconstriction elsewhere usually results in a largely unchanged or even reduced systemic resistance and the venoconstriction increases cardiac filling (Folkow, Mellander and Öberg 1961, Cobbold et al. 1964).

Since cardiac output generally increases (Folkow et al. 1968) arterial pressure is more or less raised, which then activates the arterial

baroreceptors but their reflex inhibition of the heart is almost totally suppressed (Hilton 1963 Lisander 1970). The reflex modulation of resistance to flow is however hardly at all suppressed by the defence reaction particularly not in the skeletal muscles with the result that cardiac output is enhanced and favours the skeletal muscles without too drastic pressure increases (Lisander 1970).

The hormonal adjustments in the defence reaction include release of adrenaline from the adrenal medulla (Grant *et al* 1958) and an intense activation of the ACTH-cortisol system (e.g. Folkow *et al* 1967). Furthermore associated with the renal vasoconstriction a neurogenic renin release appears to ensue (cf. Davies 1973 Zanchetti and Stella 1975) thereby involving also the angiotensin-aldosterone mechanisms.

Intermittent stimulation of the hypothalamic defence area in rats with chronically implanted electrodes leads to a gradual increase in resting blood pressure which was not observed in equally treated but unstimulated controls (Folkow and Rubinstein 1966). The importance of frequently elicited defence reactions for the gradual initiation of a raised arterial pressure level has further been demonstrated in genetically normotensive laboratory animals exposed to chronic stress (e.g. Henry Meenan and Stephen 1967 Herd *et al* 1969 Smookler *et al* 1973 Alexander 1974).

As mentioned above the labile phase of hypertension in man frequently exhibits a closely similar hyperkinetic circulation like that seen during mild defence reactions in animals. Since the question of an enhanced adrenergic activity in essential hypertension has been much debated several investigations have been designed to evaluate whether hypertensive subjects react with more intense defence reactions than normotensives when under emotional stress. Thus young patients with essential hypertension tend to increase their urinary catecholamine excretion more than normotensives during mental stress (Nestel 1969). It has also been observed that hypertensives display more pronounced tachycardia (Kalis *et al* 1957) as well as more longlasting and intense blood pressure increases when compared with normotensive controls in connection with stressful conversation or forced mental arithmetics (e.g. Brod 1963). Similar signs of a cardiovascular hyperactivity have also been reported in SHR exposed to prolonged stress (Yamori *et al* 1969).

METHODOLOGICAL CONSIDERATIONS

Experimental animals

Throughout the present experiments the spontaneously hypertensive rat (SHR) of the Okamoto strain (Okamoto 1969) was used as an experimental model for essential hypertension in man (see Introduction). Originally Okamoto and his group developed SHR by selective inbreeding of Wistar rats of the Kyoto strain choosing animals with arterial pressure levels above normal and subsequent generations were obtained through brother-sister mating. A small stock of SHR was supplied from NIH to this laboratory in 1968 and breeding has been continued here although not always with brother-sister mating. In order to diminish the extent of disturbing passenger phenomena not relevant for the hypertensive state per se

SHR hypertension develops gradually with age and mean arterial pressure is slightly above normal already at the age of 6 weeks. Thus different phases of this primary hypertension can be classified according to the age of the animals. The age around 6-10 weeks corresponds to an early what may be called prehypertensive phase. The animals show a gradual increase in pressure and may be classified as having a fully established hypertension at the age of 6-8 months. The rate of pressure rise and mean arterial pressure levels at different ages vary somewhat with different substrains and presumably also with the methods and general conditions during which the pressure measurements are performed.

In study I renal hypertensive rats (RHR) were also used. Renal hypertension was induced in 6-7 week old normotensive male Wistar rats by placing a standardized silver clip on the left renal artery during ether anaesthesia leaving the right kidney intact. About 70 per cent of the operated rats developed marked hypertension within 3 weeks after operation. Hypertensive rats (blood pressure ≥ 150 mm Hg) were used for experiments 4 weeks after operation.

Blood pressure and heart rate measurements

All pressures given throughout the present studies are mean arterial pressure as measured intra-arterially. When measured in awake animals that are left undisturbed and are properly handled the intra-arterially recorded mean arterial pressure is in all likelihood the most reliable way of estimating the true resting pressure level. The technique used in studies I, II and V was as follows. The rats were lightly anaesthetized with ether while the caudal artery was cannulated. In the animals used

for subsequent stress stimulation (studies I and II) Xylocain® containing 2 per cent of adrenaline was applied around the incision which was then sutured and the tail was bandaged with tape. These procedures were used to avoid any irritation from pain and the animals usually appeared to be fairly undisturbed by the arrangements. The rats were then allowed to wake up from the ether anesthesia and mean arterial pressure and heart rate were recorded in the freely moving animal via a long arterial cannula which was connected to a Statham pressure transducer and a Grass polygraph. No difference was obtained whether mean arterial pressure was recorded 5 or 60 minutes after the animals had woken up from ether anesthesia. Heart rate in SHR was however somewhat lower and more stable 60 minutes compared with 5 minutes after anesthesia while the normotensive control rats (NCR) were less affected in this respect.

In study III mean arterial pressure was measured in the cannulated carotid artery under standardized Nembutal® anesthesia (3 mg/100 g body weight i.p.). Tracheal cannulation was then also performed to allow free airways before the pressure was recorded. However as repeatedly tested the tracheal intubation and the cannulation of one carotid artery tended to increase the pressure level considerably even though most baroreceptor sites remained intact. Thus when measuring the mean pressures in the cannulated tail artery in awake otherwise intact animals the subsequent exposure to Nembutal® anesthesia unilateral carotid occlusion and tracheotomy led to a pressure increase of 49 ± 6 mm Hg in SHR and 25 ± 7 mm Hg in NCR. Therefore the definitely higher arterial pressures in both SHR and NCR in study III compared to the other studies is mainly a consequence of these procedures. However this did not to any greater extent increase the ratio between the mean arterial pressures in SHR and NCR which in this particular case was the relevant point. In study IV the mean arterial pressure although recorded via the tail caudal artery was measured during Nembutal® anesthesia but information concerning mean arterial pressure in the awake situation was obtained here by indirect blood pressure measurements performed prior to the actual experiment (for details see Weiss 1974).

Arrangements for acute psychological stress stimulation

In studies I and II the cardiovascular responses to different types of alerting stimuli (acute psychological stress) were evaluated in a specially designed test box. Pairs of matched rats either SHR and NCR or renal hypertensive rats (RHR) and NCR were always used in these experiments. The box consisted of two identical chambers sized

25x20x25 cm and separated by a wall into which two loud speakers were mounted and directed towards each chamber. Sawdust was placed on the floor to make the environment as familiar as possible. Three different types of alerting stimuli were used i.e. sudden flashes of bright light which was the weakest type of stimulus, loud noise and vibrations which latter proved to be the strongest alerting stimulation. Thus above the two chambers two parallel-coupled 100 W lamps were mounted which could deliver flashes of variable intensity and at random intervals. The loud noise of variable intensities and frequencies was delivered to the two chambers through their loud speakers at irregular intervals. The test box was mounted on four strings and could suddenly be vibrated by a motor driven, uncentered wheel under the box.

After tail artery cannulation the two rats were left undisturbed in the test box for 60 minutes in the sound isolated, semidark laboratory before they were exposed to any alerting stimuli. Via long polyethylene catheters connected to Statham pressure transducers placed outside the box at the level of the rats, their mean arterial pressures and heart rates were continuously followed on a Grass polygraph, using a tachygraph that was triggered by the systolic pressure rise. Each alerting stimulus lasted for 30 seconds with intervals of 10-15 minutes between stimulations.

When drugs were administered they were given via the arterial cannula dissolved in 0.4-0.5 ml of saline. Partly due to the local anesthesia the animals appeared in general to be quite undisturbed by the tail artery cannulation and sat calmly in the box chambers after an initial exploration, licking their fur and sometimes even sleeping. However occasionally rats were somewhat excited, particularly SHR and especially those which had earlier been kept socially isolated. Not infrequently they explored the thin catheters and bit them off, thus causing blood loss. In these cases the experiment was discarded. Characteristically this never happened with NCR which in general were more peaceful.

Hindquarter perfusion procedures

In studies II, III and IV the isolated hindquarter vascular beds of pairs of SHR and NCR were simultaneously perfused in order to evaluate the structural design of the resistance vessels. A detailed methodological description of this technique is given in study III as well as by Lundgren (1974) and Weiss (1974) and will therefore only be briefly outlined in this connection.

Perfusion medium

Since the paired isolated hindquarters were perfused with a non-recir-

culating plasma substitute neurohormonal or blood borne interferences with vascular tone were eliminated. As perfusate was used oxygenated Tyrode solution containing in mM/l: NaCl 136.9 KCl 2.7 CaCl₂ 1.9 MgCl₂ 1.1 NaHCO₃ 11.9 NaH₂PO₄ 0.4 and glucose 5.6 to which 3 per cent (study III) or 4 per cent (study II and IV) of Ficoll (a synthetic polymer of sucrose and epichlorohydrin - Pharmacia AB Uppsala Sweden) was added as a colloid substitute. Depending on the limited supply different batches of Ficoll had to be used and the molecular weights were either 80 000 (study III), 70 000 (study II) or 47 000 (study IV). The temperature of the perfusate reaching the preparation was 35°C pH was 7.35 and osmotic pressure around 300 mOsm/l. The viscosity of the perfusate determined in a Wells Brookfield Micro viscometer varied between 1.1 and 1.3 depending on whether 3 or 4 per cent Ficoll was used.

However these variations in perfusate composition between the different studies will not interfere with the comparison between SHR and NCR since all results are based on differences in paired experiments where occasional variations in perfusate composition or temperature will influence SHR and NCR to an equal extent. When comparing the mean values of study II and III such interferences as well as the use of the combined skin and muscle preparation of both males and females in study III probably accounts for the different absolute values obtained. Although colloids were added to the perfusate edema formation occasionally occurred towards the end of the experiment. This was especially noted in study IV where the maximally dilated vascular beds were exposed to high transmural pressures and in later phases to continuous exposure to barium ions which intensely constrict also the postcapillary resistance vessels thereby increasing capillary pressure markedly (Folkow et al 1974).

Technical arrangements

A schematic illustration of the experimental setup is given in Fig. 1. The technique which was first introduced in study III has been slightly modified afterwards but only concerning details. Thus the perfusion pressures which originally were measured via a side tube in the wide aortic cannula were later recorded in the caudal artery thereby avoiding any possible interferences in terms of a slight pressure drop along the short aortic cannula end. However in study III flow was kept constant throughout the experiment and the minor flow resistance offered by the aortic cannula was recorded after each experiment and subtracted from the presented values. By cannulating the tail artery as was done in studies II and IV the main part of the tail circulation was eliminated. Since this vascular bed like that of the feet represents mostly skin a

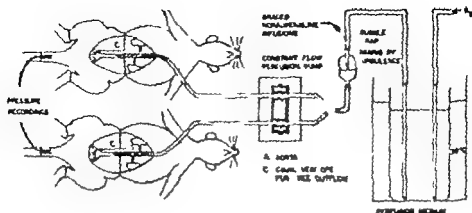


Fig. 1 Schematic illustration of the experimental arrangements during paired hindquarter perfusion used in studies II, III and IV. The oxygenated 38°C warm perfusate passes first via a common tube into which a g graded noradrenaline doses can be infused prior to a mixing chamber. The tube emerging from the mixing chamber branches into two parts which after passing through a double Harvard perfusion pump are connected to the two animals via their respective aortic cannulas (A). After passing the hindquarter vascular beds the perfusate leaves freely via the cut inferior caval veins (C). Perfusion pressures are measured via the tail (caudal) arteries.

more pure muscle preparation was obtained in studies II and IV by placing tight ligatures around the feet and tail.

Further improvement of the technique for evaluating the resistance vessel design was introduced by performing pressure-flow recordings during maximal dilatation, which was first secured by repeated injections of papaverine. For each individual experiment pressure flow curves were constructed and the resistance at maximal dilatation at different distending pressures and flow rates could thereby be calculated. Thus by presenting resistance at maximal dilatation at the higher flow rate of $30 \text{ ml/min} \times 100 \text{ g}$ as in study II, the impact of differences in vascular distensibility between SHR and NCR will also be revealed (see paper IV).

Then graded vasoconstrictions were induced by stepwise increases of noradrenaline infusion during constant flow perfusion until maximal pressor responses to NA had been induced. In study II a final addition of supramaximal amounts of barium chloride and vasopressin was added to secure a definitely maximum contractile effort of all vessels contributing to flow resistance. Then resistance curves were plotted for each animal with log NA dose on the abscissa and the pressor (resistance) responses on the ordinate (cf. study III).

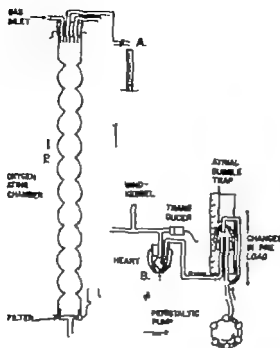


Fig 2 Schematic Illustration of the anterograde perfusion of isolated heart preparations used in study V

Isolated heart perfusion

The perfusion technique used in paper V was originally developed by Morgan *et al* (1965) and modified by Isaksson (1972) for studying cardiac metabolism. In contrast to the Langendorff preparation this method allows the heart to maintain its pumping function *in vitro*, since the perfusate enters the heart via the cannulated left atrium and leaves the heart via the aorta. For details concerning the perfusion procedure see paper V and Isaksson (1972). A schematic illustration of the perfusion apparatus is given in Fig 2. By using this anterograde perfusion of an isolated heart changes in cardiac work can be evaluated since the load on the heart can be varied by increasing either the preload or the afterload. The preload was varied by changing the level of the atrial bubble trap (see Fig 2). The afterload was basically determined by the height of a hydrostatic pressure set at 70 cm H_2O above the heart but during ventricular expulsion the afterload was somewhat raised depending on the

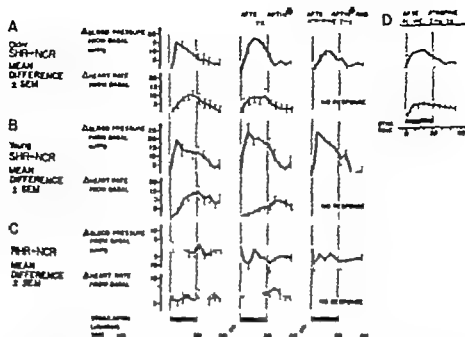


Fig. 3 Mean differences between 7 month old SHR and NCR (A) between young SHR and NCR (B) and between RHR and NCR (C) concerning heart rate and blood pressure responses during 30s exposure to vibrations and the subsequent 30s poststimulatory period. From left to right are shown the responses before any drugs are given after administration of a β -adrenergic blocking agent (Aptin®) and after both Aptin® and atropine administration. The far right section shows the situation when SHR and NCR are given atropine alone (D). The differences in blood pressure between hypertensive and normotensive animals in each group are based on the mean of the differences obtained in each paired experiment as calculated each third second. Likewise the differences in heart rate are first expressed as the mean difference in each pair of hypertensive and normotensive animals given here as percentual change from baseline. However, when given as absolute changes in heart rate the differences are equally significant. The mean of all these differences are presented as curves with SE indicated as vertical bars.

1974) showed about equal blood pressure increases as NCR. Therefore the balance between cardiac output and total peripheral resistance seems to be about equal in RHR and NCR although both vasoconstrictor and vasodilator responses are likely to be exaggerated in RHR due to the previously mentioned structural vascular changes. On the other hand in SHR there were clear signs of an increased sympathetic discharge in response to alerting stimuli also involving the heart

Other things being equal this is likely to change the balance between cardiac output and total peripheral resistance in such a direction as to cause a more pronounced increase of arterial pressure in SHR than in NCR or RHR.

The relative roles of sympathetic and vagal modulations of heart rate were judged in study I by comparing the heart rate changes after adrenergic β -receptor blockade or after atropine. After β -blockade the heart rate of SHR fell significantly more than that of NCR or RHR. With atropine administration alone heart rate increased more in NCR than in SHR. These drug effects thus suggest an increased sympathetic but a reduced vagal discharge to the heart in SHR when compared to NCR and RHR and this difference was present also when the animals were not exposed to the alerting stimulations. However the foreign environment may well imply a certain degree of stimulus and the observed differences in autonomic control between SHR and NCR might therefore be smaller during complete rest. In any case the intrinsic heart rate i.e. that after total nervous blockade to the heart by administration of both a β -blocker and atropine was slightly though significantly lower in SHR than in NCR. Therefore the mentioned shift in neurogenic cardiac control in SHR even if present during complete rest does not necessarily imply any higher resting heart rate in SHR than in NCR.

The tachycardia responses to the alerting stimuli were not only more intense in SHR but also were more consistently elicited than in NCR and RHR. In which one third of the responses were in the direction of bradycardia. This bradycardia response to alerting stimuli was not primarily a reflex adjustment to the pressure rise but must have been centrally elicited since it slightly preceded the pressure rise in most cases. Consequently NCR and RHR responded more frequently than SHR to these types of stressful stimuli with an autonomic pattern that includes vagal activations while SHR responded more consistently with sympathetic excitation combined with vagal suppression. After atropine alone all animals responded with tachycardia to the alerting stimuli used but SHR responded most intensely. This illustrates that the above mentioned bradycardia in NCR/RHR was purely vagal in origin. Furthermore when clearcut tachycardia responses occurred which were associated only with an increased cardiac adrenergic discharge these responses were more intense in SHR than in NCR. As mentioned above also the blood pressure responses to the alerting stimuli were more pronounced in SHR particularly in the young animals where the average pressure rise was 4-5 times larger and also tended to be more prolonged than in either NCR or RHR (see Fig. 3).

When taken together these results suggest an increased central reactivity in SHR for eliciting excitatory cardiovascular responses during alertness and mental stress in the direction of defence reactions. This

central hyperreactivity and apparent prevalence for sympathicotonic patterns in SHR evidently reflect a genetically linked difference from NCR and is not dependent on the high blood pressure per se. Thus it was evident also in the young prehypertensive SHR while RHR which lack genetic predisposition to high blood pressure responded like NCR.

Besides displaying more intense and somewhat more prolonged defence reactions SHR also responded more frequently with pressure and heart rate increases to the weakest alerting stimuli used indicating a lower threshold than in NCR for eliciting defence reactions in response to environmental stimuli. Since the normal environment of rats or any species probably offers frequent situations calling for enhanced alertness the above mentioned difference in central reactivity is likely to result in more frequent intense and prolonged pressure increases in SHR than in NCR. Such transiently enhanced neurohormonal discharges during daily life must imply an increased average pressure load on the SHR cardiovascular system already in prehypertensive or early phases of hypertension. This is likely to trigger a gradual structural cardiovascular adaptation (see below). Such functional and structural factors tend to potentiate each other in a way which appears to be crucial for establishing a chronic hypertensive state.

In order to test the relevance of such a proposed sequence of events SHR were in study II kept isolated from the age of weaning for 6 months in order to eliminate such environmental stimuli that are inherent in social confrontations. Although their cardiovascular responses to standardized alerting stimuli were at least as vivid as in isolated control SHR the development of hypertension was delayed or suppressed in these isolated SHR compared to age matched control SHR. In contrast the blood pressure level of similarly isolated NCR did not differ from their unisolated controls (see Fig. 4). Moreover the structural cardiovascular adaptation was correspondingly less pronounced in the isolated SHR compared with the control SHR (see also below). Therefore these findings indicate an important interaction between a genetically linked hyperreactivity concerning cardiovascular pressor responses in SHR and trivial stimuli per se, inherent in their normal environment. Together such functionally interacting elements will contribute as efficient trigger mechanisms to the gradual development of structural cardiovascular adaptation.

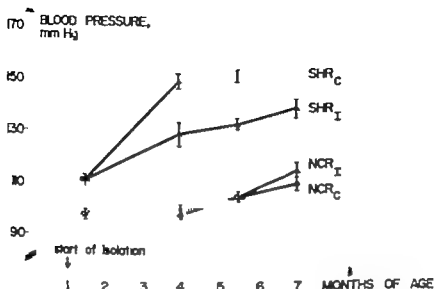


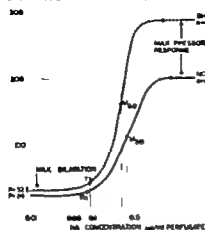
Fig. 4 Levels of mean arterial pressure as measured intraarterially during resting awake conditions (\pm SE) for control SHR (SHRC, $n = 10$), isolated SHR (SHRI, $n = 9$), isolated NCR (NCRI, $n = 10$) and control NCR (NCRc, $n = 10$) followed from the age of 1.5 months up to 7 months of age.

Structural adaptation of the resistance vessel in SHR

Study III - The evaluation of changes in the design of the resistance vessels was carried out by quantitative measurements of the pressure responses during constant flow perfusion of the hindquarter vasculature in pairs of SHR and NCR. This hemodynamic estimation of resistance vessel design was based on the analysis of the NA dose-response curves in SHR and NCR. Starting from maximal dilatation, the degree of resistance vessel constriction was gradually increased until the maximal pressor response of the resistance vessels was reached which represents their maximal contractile strength. This technique was developed in study III and utilized also in study II.

In agreement with earlier findings in hypertensive man, no resistance was demonstrated in SHR compared to NCR animals, as smooth muscle relaxation. The NA threshold dose was defined as NA concentration in the perfusate which increased resistance 25% above that at maximal dilatation which roughly corresponds to 50% shortening of completely relaxed vascular smooth muscle. NCR did not differ in this respect which showed that SHR -

COMPILED EXPERIMENTAL RESULTS

PERFUSION PRESSURE, mm Hg
(PROPORTIONAL TO FLOW RESISTANCE)

HYPOTHETICAL RESISTANCE VESSELS

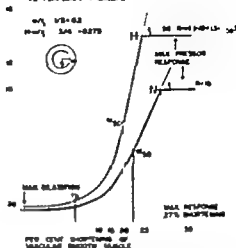
RESISTANCE PROPORTIONAL
TO PERFUSION PRESSURE

Fig 5 Left average resistance curves for SHR and NCR based on the results of 15 paired experiments from the state of maximal dilatation to maximal pressor (resistance) responses to noradrenaline. Right: mathematically deduced resistance curves for two hypothetical resistance vessels H and N where H differs from N only in that its media thickness is increased 30 per cent, encroaching upon its lumen even at complete smooth muscle relaxation. w/r_1 = ratio of wall thickness to internal radius.

smooth muscle was not hypersensitive to NA which is in agreement with findings on isolated strips of vessels (e.g. Spector et al 1969, Hallböök, Lundgren and Weiss 1971). However, both the steepness of the resistance curve and the maximal pressor response to supramaximal doses of vasoconstrictor agents were increased in SHR, i.e. the hypertensive resistance vessels exhibited exaggerated luminal reductions and an increased maximal contractile strength compared with NCR. When taken together these findings suggest an increased media thickness, partly encroaching upon the lumen even during maximal dilatation, but with a largely normal smooth muscle sensitivity to the adrenergic transmitter.

This interpretation of the experimental resistance curves (Fig 5 left part) was supported by a comparison with computed resistance curves for two hypothetical resistance vessels, one normotensive (N) and one hypertensive (H). N and H were assumed to be identical except for a 30 per cent thicker media in H, which correspondingly reduced the lumen at complete relaxation. Thus, while the wall/lumen ratio at maximal dilatation was set to 1/5 for N (cf. van Citters 1966), it was 1 3/4 for H (Fig 5 right part). For construction of the experimentally ob-

tained resistance curves in SHR and NCR the NA doses are plotted in a log scale along the abscissa. To give a comparable scale on the abscissa for the N H resistance curves the S-shaped relationship between log NA and shortening of vascular strips was utilized. From this relationship the per cent smooth muscle shortening could be plotted along the abscissa in due proportion to log NA dose (Fig 5 right part). Furthermore the maximal contractile strength of N was arbitrarily set to 10 times the resistance (or pressure) at maximal dilatation. With an equal ability for tension development per unit contractile element in N and H but with 30 per cent thicker media in H its maximal strength is 30 per cent greater than in N. When a correction according to Laplace's law for the smaller radius in H is taken into account (see Fig 5 right part) a 40 per cent higher maximal pressor response is obtained in H compared with N.

NA sensitivity is assumed to be equal in N and H in accordance with findings on arterial strips from SHR and NCR (Clineschmidt et al 1970, Hallböök, Lundgren and Weiss 1971). Furthermore the contractions are considered to be initiated from the outermost media layer since the adrenergic neuroeffector junctions in precapillary resistance vessels are concentrated to this site (e.g. Norberg and Hamberger 1964). Also the great majority of α -receptors appear to be situated here judging from *in vitro* responses of vascular smooth muscle to exogenous NA (Johansson et al 1970). When applied to the model vessels it implies that inner wall layers become displaced towards the lumen when contraction occurs and the thicker the wall the more exaggerated the resistance increase will be. It is therefore clear that a resistance vessel design like that in H will proportionally raise the baseline, i.e. the resistance at maximal dilatation, cause an increased steepness of the resistance curve and raise the maximal pressor response at normal functional characteristics *per se* of the effector cells in relation to N. It is seen from Fig 5 that the resistance curves of the two model vessels in their mutual relationships closely mirror those of the NCR SHR resistance vessels.

In fact should the altered characteristics of the SHR resistance curve be ascribed to other factors one must not only assume a simultaneous involvement of several different changes but also disregard all hemodynamic consequences of the well-documented wall thickening in hypertensive precapillary vessels. For example an increased muscle sensitivity to NA would only cause a parallel displacement of the resistance curve to the left but not alter its configuration. An increased tension development per unit contractile element being *per se* unlikely as the contractility of larger SHR arteries is. If anything decreased (e.g. Spector et al 1969) cannot explain the increased resistance at maximal dilatation. A reduced size or number of resistance vessels but with an unchanged wall/lumen ratio could as suggested by Hutchins

and Dornell (1974) explain the increased resistance at maximal dilatation but certainly not the increased maximal contractile strength of the SHR resistance vessels which has been tested in model experiments (unpublished results). Likewise increases of wall/lumen ratio by addition of noncontractile elements or water logging (Tobian and Binion 1952) cannot explain the markedly increased maximal contractile strength of the SHR resistance vessels since this calls for a larger mass of contractile elements.

Therefore the present experimental data (study III) clearly indicate that the SHR resistance vessels exhibit a hemodynamically most important increase in wall/lumen ratio which is mainly the result of a relative thickening of the muscle sheath. This changed resistance vessel design may in fact explain their hyperreactivity and ability to maintain a raised resistance which does not necessitate any tonic increase of smooth muscle activity. Furthermore in a more recent study it could be demonstrated that only the precapillary resistance vessels in SHR show exaggerated resistance responses and signs of altered design while capillaries and postcapillary vessels appear largely normal in dimensions, design and reactivity (Folkow et al 1974).

Study IV - A partly different approach for exploring the altered vascular design in SHR was used in study IV. Here the distensibility of the resistance vessels was studied in SHR and NCR both at maximal dilatation and at increasing levels of smooth muscle contraction. Earlier studies demonstrating reduced vascular distensibility in hypertension have mostly dealt with large arteries (e.g. Feigl, Peterson and Jones 1963, Greene et al 1966) whereas the present study was designed to study the true resistance vessels. Paired hindquarter perfusions were performed in a similar way as in studies II and III. During maximal dilatation or at stable levels of smooth muscle activity obtained by suitable concentrations of BaCl_2 or NA in the perfusate flow was altered in a randomized fashion and the resulting pressure changes were recorded. According to Poiseuille's law resistance (R) varies inversely with the fourth power of the internal radius and hence the internal radius of the average resistance vessel was arbitrarily expressed as $\sqrt[4]{1/R}$. The distensibility of the resistance vessels was thus compared in SHR and NCR by relating the per cent increase of internal radius for a 10 mm Hg change in average distending pressure which was calculated as the mean of the arterial and venous pressures.

During maximal dilatation when nonmuscular wall elements are likely to offer the main resistance to the distending pressure the distensibility of the SHR resistance vessels was significantly lower (some 11 per cent) than that of the NCR ones. During smooth muscle activity the shortened actomyosin complexes offer the main resistance to distension

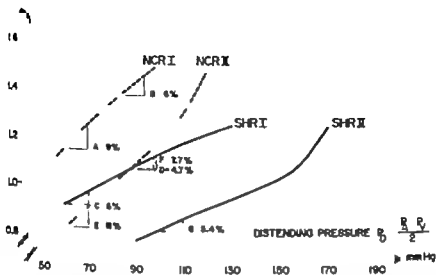


Fig. 6 The relationship between average distending pressure (P_D) and calculated internal radius (r_i) for the average resistance vessel in 3 representative pairs of SHR and NCR. Curves labelled I represent the situation when flow resistance was kept increased about 3 times above that at maximal dilatation induced by barium chloride while curves labelled II represent a vascular tone approximately 5 times that at maximal dilatation. Comparisons are made between NCR and SHR concerning resistance vessel distensibility ($\Delta r_i \% / \Delta P_D$) at the same initial P_D (A and C) at the same initial r_i (E and G) and when both P_D and r_i are initially the same (F and D). See also text.

since they tend to unload other wall elements (cf. van Citters, Wagner and Rushmer 1962). Therefore the distensibility of the SHR NCR resistance vessels was also compared during stable levels of vascular smooth muscle tone which largely corresponded to those present in resting skeletal muscle *in vivo*. During smooth muscle contraction the difference in resistance vessel distensibility between NCR and SHR increased about threefold compared with the difference seen during maximal dilatation.

When comparing the distensibility of the NCR SHR resistance vessels distending pressure, smooth muscle activity and internal radius should ideally all be kept as equal as possible in the two circuits. However this is impossible because of the increased reactivity of the SHR precapillary resistance vessels. For such reasons resistance vessel distensibility was first compared at equal smooth muscle activity and internal radius but at different distending pressures (E and G, Fig. 6). Then the comparison was repeated when smooth muscle activity and distending pressure were equal but at different internal radii (A and C, Fig. 6). Lastly a

comparison was made when both distending pressure and internal radii were the same in NCR and SHR (F and D Fig 6) in which case less BaCl_2 (or NA) had to be given to the hyperreactive SHR precapillary resistance vessels thus implying a lower level of vascular smooth muscle activity in SHR.

The compiled results of 14 experiments show that during contraction the SHR resistance vessels are some 35-45 per cent less distensible than the NCR ones regardless of the mode for comparison. Thus these results strongly suggest that particularly the media component of the SHR resistance vessels is increased in bulk. Especially comparisons like that shown as F D in Fig 6 definitely exclude that the difference in behavior and reactivity between SHR and NCR resistance vessels is merely due to some difference in smooth muscle activity.

Furthermore these results also illustrate the raised contractile strength of the SHR resistance vessels here reflected by their decidedly higher yield pressures when exposed to such high distending pressures that the contracted vascular smooth muscles were forced to yield. This was revealed as an abrupt and steep decrease in resistance in Fig 6 shown as an increase of calculated internal radius when the distending pressure exceeded a critical level which was considerably higher in SHR than in NCR. It follows that these results concerning the distensibility of the resistance vessels lend further strong support to the results and conclusions in study III.

Study II - The extent of change in structural vascular design seems to be largely proportional to the increased arterial pressure level in SHR indicating a close correlation between pressure load and structural adaptation of the resistance vessels. Studies in renal hypertensive rats show that the duration and extent of blood pressure increase determine the degree of structural vascular adaptation which seems to be fully established within 3-4 weeks in young animals (Lundgren 1974). Furthermore a local or general reduction of the functional pressure load prevented the development of structural changes in the SHR resistance vessels or caused regression of already established changes (Folkow et al 1971 Weiss 1974).

Towards such a background the resistance vessel design was hemodynamically analyzed in a group of SHR which from weaning had been isolated from social confrontations. This change of environment is likely to reduce the frequency and extent of intermittent increases in central neurohormonal discharge to the cardiovascular system. In SHR the extent of these discharges seems to be exaggerated for genetical reasons (study I). The hemodynamic analysis of the hindquarter vascular bed in these animals was carried out at 7 months of age after prolonged isolation and gave the following results. The slope of the resistance curves reflecting

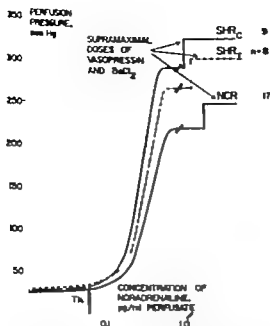


Fig 7 Average resistance curves of constant flow perfused hindquarter vascular beds from control SHR (SHR_C) SHR kept isolated from social contacts for 5 1/2 months (SHR_I) and paired NCR. The curves show the mean resistance (pressure) changes from maximal vasodilatation to maximal smooth muscle activation as induced by graded noradrenaline infusions and finally by adding supramaximal doses of vasopressin and $BaCl_2$. Th indicate the threshold NA dose.

the wall/lumen ratio of the resistance vessels and the maximal pressor response reflecting their contractile strength were both significantly reduced in isolated SHR compared to the unisolated control SHR. Resistance at maximal dilatation did not differ significantly (Fig 7). Also the structural adaptation of the left ventricle was proportionally less pronounced in the isolated SHR compared with the control SHR as judged by their significantly lower per cent left ventricular weight of body weight.

These results therefore illustrate the important consequence of functionally induced differences in average arterial pressure level for the design of the cardiovascular high-pressure sections. Thus any reduction in environmentally induced neurogenic pressor influences which in SHR seems to be amplified due to a central autonomic hyperreactivity to psychogenic stimuli implies a reduction of the average functional pressure load and hence a less pronounced structural adaptation with a corre-

spondingly lower resting arterial pressure. Thus the potential vicious circle inherent in the interaction between functional excitatory influences and secondary structural changes in SHR can to some extent be counteracted by limiting such exogenous stimuli which tend to reinforce the inherent hyperreactivity of higher autonomic centres.

Structural adaptation of the heart in SHR

The establishment of left ventricular hypertrophy seems to take place very early in SHR and increases in extent as hypertension proceeds (e.g. Sen et al 1974, Farmer et al 1974). In the present study (V) the consequences of this structural cardiac adaptation for the Frank-Starling relationship was investigated in isolated perfused heart preparations from young and adult SHR. The results show that the hypertrophied hearts from adult 7 month old SHR require a higher diastolic filling pressure to produce the same left ventricular stroke volumes or stroke work than hearts from age matched NCR when compared at the normal range of left ventricular filling pressure between 5 and 10 cm H₂O (Fig. 8 left part). At higher filling pressures this difference was no longer apparent since the NCR Starling curve tended to level off at filling pressures when that of the SHR ones was still ascending.

This shift to the right of the SHR Starling curve could not be ascribed to any compensatory luminal change because end diastolic volumes were largely the same as in age matched NCR compared at equal filling pressures. These latter measurements were performed on isolated SHR and NCR hearts arrested by and filled with ice-cold Krebs solution during exposure to different filling pressures while left ventricular volume (weight) was continuously recorded. The obtained curves relating pressure and calculated intra ventricular volume did not differ significantly between SHR and NCR although the percentual change in left intra ventricular volume per unit change in pressure tended to be somewhat lower in SHR.

The measured end-diastolic volumes and the wet weight of the left ventricles also allowed for a calculation of left ventricular geometry and wall/lumen ratio at different end diastolic filling pressures by assuming the ventricle to be a sphere. As a result of the thicker wall of the SHR left ventricle the elongation of the outer and mean circumferences representing the length of the outer and mean myocardial elements were shown to be significantly smaller in SHR than in NCR upon equal increases in filling pressure. Moreover calculations of wall tension per unit tissue mass which determines the degree of stretch on the average myocardial fibre (Starling 1918) revealed that the average muscle fibre of the SHR left ventricle was exposed to a significantly

SHR NCR 30 WEEK OLD

SHR NCR 6 WEEK OLD

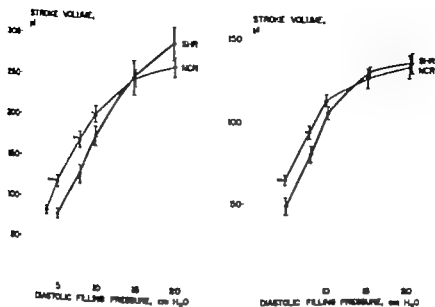


Fig 8 Relationship between diastolic filling pressure and stroke volume (Frank-Starling relationship) for 30 week old SHR and NCR (left part) and for 6 week old SHR and NCR (right part)

lower tension at a given end-diastolic filling pressure compared with NCR. In other words the more the left ventricular wall/lumen ratio is increased by hypertrophy the less pronounced is the average myocardial diastolic prestretching for any given increase in filling pressure even though left ventricular distensibility as judged by the luminal changes is not necessarily changed.

Comparisons of the Frank-Starling relationship of hearts from NCR and SHR 6 week old and representing early phases of hypertension were also performed. Already at this age mean arterial pressure and left ventricular weight/body weight ratio were significantly increased in SHR although far less so than in adult SHR. In the lower range of diastolic filling pressures (5-10 cm H₂O) the stroke volumes were also significantly reduced in these young SHR compared to age matched controls although to a less extent than in adult SHR (Fig 8 right part). However the stroke work i.e. the product of left ventricular output and the pressure built up during ejection was then only slightly decreased in SHR and at higher filling pressures (15-20 cm H₂O) the SHR left ventricles exhibited significantly greater stroke work than the NCR ones. This indicates a relatively greater flow acceleration induced by the SHR left ventricle during ejection since stroke volumes and heart rates at

these higher filling pressures were largely equal in SHR and NCR. Thus maximal cardiac work performance of the young SHR was significantly greater than that of matched controls which probably reflects the increased bulk of cardiac muscle in SHR already at this age. In older SHR the maximal left ventricular work performance could not be evaluated since their hearts had not reached the peak of the Starling curve at the highest filling pressures tested.

At a diastolic filling pressure of 8 cm H₂O the young SHR having a 40 per cent increase in the ratio left ventricular dry weight to body weight displayed a 10-13 per cent reduction in stroke volume compared with age matched NCR. Whereas the adult SHR with a 80 per cent increase in left ventricular weight/body weight showed a 20-25 per cent reduction in stroke volume compared with matched NCR. Thus with the progression of hypertension the Frank-Starling relationship of the progressively more hypertrophied left ventricle becomes reset so that a higher end diastolic filling pressure is required for the same stroke volume as in matched normotensive controls.

When applying these findings to the in vivo situation where neurogenic influences are superimposed on the heterometric autoregulation of cardiac performance (Sarnoff and Mitchell 1962) the maintenance of equal left ventricular stroke volumes in NCR and in SHR with established hypertension would call for a somewhat increased sympathetic drive to the capacitance side and/or to the heart in SHR. Dependent on the age-linked progression of hypertension and left ventricular hypertrophy the presence of an increased sympathetic influence may in young SHR result in an increased stroke volume and cardiac output compared with controls. This hemodynamic pattern often characterizes e.g. labile hypertension in man. On the other hand for older SHR with more developed cardiac hypertrophy equally increased sympathetic discharge may imply a largely normalized stroke volume and cardiac output which is the general finding in established hypertension in man.

GENERAL DISCUSSION

As outlined in Results and comments the hyperreactivity of spontaneously hypertensive rats (SHR) to alerting or stressful stimuli is of central origin. It reflects a response pattern where the cardiac acceleration involves both an increased sympathetic and decreased vagal tone which is characteristic of the hypothalamic defence reaction (study I). These centrally induced cardiovascular responses to the stressful stimuli utilized ~~are~~ not only to be quantitatively exaggerated in SHR but also to some extent qualitatively different in SHR and NCR. Thus neurogenic adjustments characterizing the defence reaction seem to be dominating in SHR while NCR exhibits some variability between individuals with initial bradycardia responses occurring in about 35 per cent of the cases. Such individual variations concerning both changes in heart rate and skeletal muscle vasodilatation have also been observed in awake normotensive cats when exposed to different types of emotional excitement (Zanchetti Baccelli and Mancini 1971). It appears as if normally a spectrum of differently balanced autonomic response patterns are available where any one of them can be engaged depending on individual circumstances and type of stressful stimuli.

The central hyperreactivity to alerting stimuli in SHR appears to have largely the same consequences for the cardiovascular system as when normotensive rats are exposed to environmental stimuli that are enhanced in extent, frequency and duration. Several reports have demonstrated that prolonged exposure to stressful or socially conflicting stimuli can induce more or less persistent increases in arterial pressure even in genetically normotensive laboratory animals (Henry, Meehan and Stephens 1967; Hard *et al.* 1969; Smookler *et al.* 1973; Alexander 1974). In the present study (II) a prolonged elimination of direct social confrontations (even though some contacts with the other animals were still present by way of e.g. sound and smell signals) partly suppressed the development of hypertension in SHR. However they still exhibited the characteristically exaggerated cardiovascular responses to standardized alerting stimuli. Thus their environmental situation was aimed to be a contrast to that of genetically normotensive animals when exposed to prolonged intensification of environmental stimulations.

It therefore seems obvious that enhanced and often repeated cardiovascular pressor responses to emotional stimuli, whether induced by an inherent central hyperreactivity to such stimuli as in SHR or by increasing their intensity, duration and frequency in normal animals, can be of great importance for the initiation of hypertension. However the SHR hypertension, which is generally acknowledged to have a polygenic background (cf. Okamoto 1972) seems to be more stable and pronounced

these higher filling pressures were largely equal in SHR and NCR maximal cardiac work performance of the young SHR was significantly greater than that of matched controls which probably reflects the increased bulk of cardiac muscle in SHR already at this age. In old SHR the maximal left ventricular work performance could not be tested since their hearts had not reached the peak of the Starling curve at the highest filling pressures tested.

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When applying these findings to the *in vivo* situation where nongenetic influences are superimposed on the heterometric autoregulation of cardiac performance (Sarnoff and Mitchell 1962) the maintenance of equal left ventricular stroke volumes in NCR and in SHR with established hypertension would call for a somewhat increased sympathetic drive to the capacitance side and/or to the heart in SHR. Depending on the age-linked progression of hypertension and left ventricular hypertrophy the presence of an increased sympathetic influence may in young SHR result in an increased stroke volume and cardiac output compared with controls. This hemodynamic pattern often characterizes early labile hypertension in man. On the other hand for older SHR with more developed cardiac hypertrophy equally increased sympathetic discharge may imply a largely normalized stroke volume and cardiac output which is the general finding in established hypertension in man.

hypertension (e.g. Eich et al 1966). It may be questioned whether this gradual transfer in hemodynamic balance is a reflection of some adaptive or/and compensatory process that also involves a change of cardiovascular design. Here the gradual development of the well-known left ventricular hypertrophy and of structural changes also in other high-pressure sections of the cardiovascular system i.e. arteries and precapillary resistance vessels (cf. Folkow et al 1973-1974) is likely to be of importance.

Such structural adaptations constituting a common mesenchymal response to changes in the mechanical stress applied (cf. Liebow 1963 Rodbard 1970) mainly take the form of an increased bulk of contractile tissue at least in early stages. In the resistance vessels such changes are generally associated with a luminal narrowing that is present even at complete smooth muscle relaxation as shown by hemodynamic studies in human essential hypertension (Folkow 1956; Folkow, Grimby and Thuleius 1958; Conway 1963; Sivertsson 1970). Furthermore the present results in SHR (studies III-IV) can hardly be explained by any other mechanism than by the presence of a relative increase in media thickness that partly interferes with the lumen of the precapillary resistance vessels. These changes can be very rapidly established i.e. in 2-3 weeks after gross increases in pressure load as studied in young rats exposed to renal hypertension (Lundgren 1974). In addition sustained pressure reductions whether induced by antihypertensive treatment in SHR (Weiss 1974) or by renal artery declipping in renal hypertensive rats (Lundgren 1974) lead to a rapid and considerable regression of the structural changes in both resistance vessels and heart at least in young animals. Also the present results (study II) illustrate the close correlation between average pressure load and cardiovascular structural adaptation when comparing ordinary SHR, isolated SHR and NCR concerning the relationships between arterial pressure and degree of left ventricular hypertrophy and media hypertrophy in the resistance vessels.

These adaptations in precapillary vessel design are closely balanced in extent by the change in pressure load. Thus it constitutes a morphological and "structural autoregulation". Thus it constitutes a morphological and consequently more slowly developed counterpart to the functional autoregulation confined to the precapillary resistance vessels (e.g. Folkow and Öberg 1961) which is caused by adjustments in smooth muscle activity to acute pressure changes. Also the structural autoregulation implies a raised precapillary resistance in response to regional pressure increases and *vice versa*. However since it is a matter of a change in vessel design the structural autoregulation is slower in development and does not necessitate any change in smooth muscle activity to maintain the altered resistance level.

It appears as if the structural adaptation of the cardiovascular

high pressure sections may be somewhat more extensive in SHR than in genetically normotensive rats provoked into a g secondary renal hypertension (RHR). Thus when comparing the extent of alterations in left ventricular weight and resistance vessel design in RHR and SHR where both the degree and duration of hypertension were roughly the same, the structural changes appear to be more pronounced in SHR (cf Lundgren 1974). Moreover comparisons of resistance vessel design in NCR and in immunosympathectomized SHR point in the same general direction (Folkow et al 1972). In addition compared to NCR young prehypertensive SHR show an increased arterial incorporation of proline indicating a higher mesenchymal metabolic turnover in the SHR vasculature already at a stage where arterial pressures are largely similar in SHR and NCR (Yamori 1974).

When taken together such findings indicate that the SHR resistance vessels, arteries and heart might for genetical reasons be more prone to adapt structurally to an increased pressure load than genetically normotensive rats. Whether such a possible difference is inherent in cardiac and vascular structures per se or is mainly a consequence of a changed trophic influence of a g hormonal or neurogenic origin is unknown. Whichever its exact background such an inherently increased tendency of the cardiovascular system to adapt structurally to increases in load may together with the previously mentioned central hyperreactivity in SHR form dominating genetic elements for the establishment of primary hypertension in SHR.

As outlined above the structurally increased wall/lumen ratio in live resistance vessels implies first a raised "baseline" from which vasoconstriction is initiated and second exaggerated vasoconstrictor and vasodilator responses for given changes in smooth muscle length. While increased pressor responses are well documented in both human and SHR hypertension increased depressor responses have mostly been regarded to reveal increased preexisting excitatory influences. However it is clear e.g. from the resistance curves in Fig 5 that equal reductions in smooth muscle activity in hypertensive and normotensive resistance vessels lead to exaggerated resistance reductions in the live ones even if both start from the same level of smooth muscle activity.

Interventions aiming at the elimination of sympathetic tone in SHR lead to a greater pressure fall in SHR than in NCR and low pressure levels are obtained after e.g. ganglionic pithing (Shibayama, Mizogami and Sakabe 1971, Iriuchijima 1973). This has often been taken as strong evidence of an increased sympathetic activity in SHR. However arterial pressure is not only dependent on peripheral resistance but also on cardiac output and therefore will be altered to a different extent in SHR and NCR upon a

Furthermore the resistance reduction is likely to be exaggerated in SHR thanks to the presence of structural changes in its resistance vessels. Careful measurements of cardiac output and mean arterial pressure before and after pithing in SHR and NCR with calculations of peripheral resistance show that the largely equalized pressures after pithing were the result of a higher resistance but = lower cardiac output in SHR compared with NCR (Albrecht et al 1975). The greater pressure fall in SHR was thus determined in part by a greater resistance reduction which probably reflects their exaggerated resistance responses as illustrated in Fig. 5. However in part it was also due to a percentually greater reduction of cardiac output. The presence of a raised resistance even after complete denervation when the marked pressure fall leads to nearly maximal vasodilatation in both SHR and NCR confirms earlier results showing a structurally determined generalized resistance increase in SHR (Folkow et al 1970).

The lower cardiac output in SHR after pithing may reflect structural differences in the low-pressure sections i.e. in the capacitance vessels or/and in the heart itself since cardiac performance upon denervation is determined mainly by the end-diastolic filling (cf. Sarnoff and Mitchell 1962). The SHR capacitance vessels at least in the hind-quarters seem to be largely unchanged compared with those of NCR (Folkow et al 1974). Therefore it seems more likely that the obvious left ventricular hypertrophy in SHR considerably influences the relationship between diastolic filling and cardiac performance which was explored in isolated perfused heart preparations from SHR and NCR in study V.

In this study the Frank-Starling relationship of the isolated SHR left ventricle was shown to be displaced to the right of the NCR one in the lower physiological range of filling pressures. This displacement was not a consequence of cardiac dilatation since end-diastolic volumes were about equal in the arrested left ventricles of adult SHR and NCR. Furthermore when left ventricular compliance was determined as percentual change of intraventricular volume per unit change of end-diastolic pressure the SHR and NCR hearts did not differ significantly. However it should be stressed that according to Laplace's law the thicker wall of the SHR left ventricle implies a lower tangential tension per unit wall layer for a given transmural pressure. Consequently for equal filling pressures the stretching of the average muscle fibre becomes lower in the SHR left ventricle than in the NCR one.

For such reasons the lower stroke volume in SHR appears to be primarily a consequence of the Frank-Starling law which implies that the end-diastolic fibre length rather than the end-diastolic pressure determines the stroke volume. It is in agreement with such a view that stroke volumes were $\sim 20\%$ to be proportionally more reduced in adult

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SHR with their more advanced left ventricular hypertrophy than in young SHR. Such structurally determined changes of the Frank-Starling relationship may also be of considerable importance in the *in vivo* situation. For example, cardiac output measurements in SHR of different ages indicate a decrease in stroke volume with the progression of hypertension (Pfeffer and Frohlich 1973) although in this case possible differences in superimposed neurohormonal influences must also be taken into account.

Concerning the situation in man, cardiac output is elevated or normal in early hypertension and at the same time there are often signs of an increased neurogenic activity to the heart (for ref. see Julius and Schork 1971 and Introduction). On the other hand, established essential hypertension in man is characterized by left ventricular hypertrophy, a largely normal cardiac output, but an increased resistance. Such a gradual transfer of the hemodynamics along with the development of hypertension may to a great extent be due to the presently studied interaction between functional initiating elements and the progressive structural adaptation of cardiovascular high-pressure sections.

Thus, SHR display a genetically determined central hyperreactivity for eliciting defence reactions in response to environmental stimuli. Consequently, such intermittent and neuro-hormonally mediated pressure increases tend to be more frequent, pronounced and prolonged in SHR, thereby raising the average blood pressure level. Furthermore, these pressor bursts are only poorly counterbalanced by the arterial baroreceptor reflexes, since these are considerably suppressed by the defence reaction (cf. Hilton 1963, Lissander 1970). Moreover, also the long-term pressor regulating function of the kidneys (cf. Guyton et al. 1972) is likely to be overruled in connection with defence reactions, partly because these reactions involve renal vasoconstriction and cause an acute reduction of urine formation (Feigl, Johansson and Lefving 1964) and partly because of the concomitant renin-angiotensin-aldosterone release (e.g. Zanchetti and Stella 1975).

Along with the rise in average pressure within the high pressure cardiovascular sections, the earlier mentioned structural autoregulation of the systemic precapillary resistance vessels is gradually initiated. This structural autoregulation involves also the renal vascular bed (cf. Folkow et al. 1971) which in a more definite way will reset the long-term pressure regulating function of the kidneys, since such a change in resistance vessel design implies that a higher preglomerular resistance can be maintained by a given smooth muscle activity. Similarly, by reducing the distensibility of larger arteries, such structural adaptations seem to contribute also to the resetting of the arterial baroreceptors (Aars 1969). Also, the left ventricular hypertrophy may be of great importance for the gradual shift in hemodynamics along with the development of hypertension, since it tends to reduce left ventricular

stroke volume for a given filling pressure according to the present findings. If no compensatory influences of e.g. neurogenic origin are superimposed.

In other words, these structural changes of the cardiovascular high-pressure sections have an inherent tendency to shift an early hyperkinetic circulatory state into one characterized by fairly stabilized resistance. Increase in association with a normal cardiac output and stroke volume. Such a gradual shift in hemodynamics may start quite early in primary hypertension, since the first clear signs of structural autoregulation of the resistance vessels can be found already at the age of 10-11 weeks in male SHR (unpublished) and the increase of left ventricular wall mass can be detected even earlier (e.g. Sen et al 1974). Furthermore, already within 2-3 weeks after induction of renal hypertension in rats, left ventricular hypertrophy and resistance vessel adaptation are largely completed (Lundgren 1974). Concerning man, young conscripts to military service who exhibit modestly elevated mean arterial pressure show signs of mild structural autoregulation of their resistance vessels (Sivertsson, Sannerstedt and Lundgren 1975). It is therefore possible that the raised resistance in relation to the increased cardiac output in the hyperkinetic state of early hypertension in part reflects an early involvement of such a structural adaptation.

In general, the present results illustrate how the establishment of chronic hypertension calls for the interaction of initiating functional elements and secondary structural changes, where the latter appears to be the important common denominator and a prerequisite for chronic hypertension in general. The initiating elements appear to be mainly constituted by central neurohormonal influences in the spontaneously hypertensive rat of the Okamoto strain (SHR). Concerning essential hypertension in man, a similar train of events is likely to occur, but here the initiating factors may be more heterogeneous than in the purebred SHR.

SUMMARY AND CONCLUSIONS

The present experiments were performed in order to elucidate the relative roles and temporal interaction between central neurogenic mechanisms and adaptive changes in cardiovascular design in primary hypertension as studied in spontaneously hypertensive rats (SHR). The results can be summarized as follows:

- I SHR exhibited enhanced cardiovascular responses to acute alerting stimuli compared to normotensive control rats (NCR). Also after adrenergic β -receptor blockade SHR responded with enhanced and clearcut tachycardia which were abolished after atropine. However there were no signs of any changed cardiac effector sensitivity in SHR in response to e.g. graded nerve stimulation. This points to a central hyperreactivity in SHR for eliciting defence reactions which besides an increased sympathetic drive to the heart also includes a central inhibition of cardiac vagal tone. Also young nearly prehypertensive SHR responded with enhanced defence reactions but not renal hypertensive rats. This suggests that the central hyperreactivity in SHR is a genetically linked factor predisposing for hypertension and not a consequence of the high blood pressure per se.
- II When separated from social confrontations by means of isolation from the time of weaning the development of hypertension was delayed and suppressed in SHR compared with unisolated control SHR. However the cardiovascular responses to acute psychological stress stimulations were equally pronounced in the isolated SHR as in SHR controls. The results thus stress the importance of the interaction between environmental influences and inherently exaggerated cardiovascular responses to such stimuli for the development of hypertension in SHR.
- III By hemodynamic analysis of the hindquarter vascular beds of SHR and NCR an altered design of the resistance vessels in SHR was revealed. Thus resistance responses to graded noradrenaline (NA) infusions resulted in exaggerated resistance responses in SHR which also exhibited an increased resistance at maximal dilatation and a raised maximal pressor response but an unaltered threshold sensitivity to NA. These data suggest an increased media thickness encroaching upon the lumen of the resistance vessels also during complete smooth muscle relaxation. This altered vascular design is according to later studies confined to the precapillary vascular sections and seems to be the result of a rapid local adaptation to increases in average pressure load and will by its mere presence tend to increase flow resistance and vascular reactivity in hypertension.

- IV Comparisons of the distensibility of the resistance vessels in SHR and NCR lent further strong support to the presence of an increased media thickness in the SHR vessels. Thus they were clearly less distensible than the NCR ones both during maximal dilatation and particularly during equal levels of smooth muscle activity.
- V The hemodynamic consequences of an increased left ventricular wall thickness was investigated in isolated SHR and NCR hearts. It was demonstrated that with the progression of hypertension and consequent left ventricular hypertrophy increased levels of end-diastolic filling pressure were required in the hypertrophied SHR left ventricles to produce the same stroke volumes as in NCR hearts. The Frank-Starling relationship of the SHR left ventricles was shifted to the right in the lower physiological range of diastolic filling pressures as a result of the reduced diastolic compliance of the thickened wall. Already in 6 week old SHR with only moderate left ventricular hypertrophy such a displacement of the Frank-Starling relationship was clearly evident but it was proportionally less pronounced than in adult SHR.

Compiled these results suggest that primary SHR hypertension may be initiated by a central hyperactivity for eliciting defence reactions. Environmental stimuli also seem to play a crucial role since the cardiovascular pressor response to the alerting stimuli of daily life presumably will be amplified due to the central hyperactivity to such stimuli. As a result of such intermittent pressor influences the average pressure load on the cardiovascular system will be raised in SHR thereby triggering structural cardiovascular adaptations of the high-pressure sections which seems to be a prerequisite for the establishment of a chronic hypertensive state. It is further suggested that the progressive shift in hemodynamic balance between cardiac output and resistance along with duration and severity of hypertension is mainly due to the above mentioned structural cardiovascular adaptation. Thus the structural autoregulation characterizing the precapillary resistance vessels will contribute to the rise in peripheral resistance while the hypertrophic thickening of the left ventricle will tend to reduce cardiac output though both effects can be considerably modulated by superimposed neurogenic influences of central or reflex origin.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 425

ON THE MECHANISMS

An experimental study in the cat with special reference to the
facial nucleus

by

JAN MARTNER

ACTA PHYSIOLOGICA SCANDINAVICA
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An experimental study in the cat with special reference to the
solstitial nucleus

JAN MARTNER

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 423

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CEREBELLAR INFLUENCES ON AUTONOMIC MECHANISMS

An experimental study in the cat with special reference to the
fastigial nucleus

by

JAN MARTNER

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This summary is based on studies reported in the following papers:

- I *Interaction between the fastigial pressor response and the defence reaction*
B Lisander and J Martner *Acta physiol scand* 1973 87 359-367
- II *Influences on colonic and small intestinal motility by the cerebellar fastigial nucleus*
J Martner *Acta physiol scand* 1975 In press
- III *Influences on the defecation and micturition reflexes by the cerebellar fastigial nucleus*
J Martner *Acta physiol scand* 1975 In press
- IV *Effects on gastric motility from the cerebellar fastigial nucleus*
B Lisander and J Martner *Acta physiol scand* 1975 In press
- V *Integrated somatomotor, cardiovascular and gastrointestinal adjustments induced from the cerebellar fastigial nucleus*
B Lisander and J Martner *Acta physiol scand* 1975 In press

The papers are referred to in the text by their Roman numerals

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INTRODUCTION

Our knowledge of cerebellar physiology has in the last decade been greatly expanded. Thus the interactions between the neurons constituting the cerebellar cortex have been revealed as have their afferent and efferent connections (for ref. see Eccles, Ito and Szentagotai 1967) which has meant a firmer ground for the development of theories concerning cerebellar function (e.g. Eccles 1969, Marr 1969, Ito 1970, Glibert 1974). The experimental studies forming the basis of present concepts of cerebellar function have mainly considered the somatomotor mechanisms. Moreover the most obvious disturbances observed upon cerebellar lesions whether experimentally induced or brought about by disease are those of the somatomotor control. It is therefore understandable that cerebellar influences on autonomic mechanisms have generally come in the background and have sometimes even been more or less overlooked.

However, it has long been known that autonomic responses can be induced from the cerebellum and in recent years several studies have lent considerable support to the idea that the cerebellum is indeed of considerable importance also for the regulation of autonomic mechanisms as will be outlined below.

CEREBELLAR ANATOMY AND FUNCTION

For a detailed description of cerebellar organization the reader is referred to monographs by Jansen and Brodal (1954) and by Eccles *et al* (1967) and here only some principles of particular relevance for the present study will be briefly outlined.

On phylogenetic grounds the cerebellum was earlier usually divided into the archicerebellum, paleocerebellum and neocerebellum. However, recent anatomical and functional findings better correspond to a sagittally oriented organization according to which the cortex consists of a medial zone, the vermis, bordered by the intermediate or paravermal zones which are laterally flanked by the hemispheres. The great majority of the cerebellar autonomic effects observed have been elicited from the medial zone, the vermal cortex, and from its efferent projections as will be further outlined below.

The neuronal organization of the cerebellar cortex is strikingly uniform independent of anatomical localization or functional involvement. The cortex can be divided into three layers where the Purkinje cells form the middle or ganglionic layer which separates the superficial or molecular layer from the deeper or granular one. In the latter, deep layer the small excitatory granule cells are found together with the inhibitory Golgi cells. The superficial molecular layer contains the stellate and the basket cells which like the Golgi cells function as inhibitory interneurons.

The afferent input to the cerebellar cortex is delivered via the climbing fibres and the mossy fibres. These afferents convey an abundance of information to the cortical integrator at the same time as they via

axon collaterals exert an excitatory drive on the cerebellar nuclei (see below). This afferent information to the cerebellum is derived from a variety of sources including the motor cortex and various subcortical motor neuron pools, bulbar and spinal reflex integration centres and virtually all types of peripheral sensory receptors, somatic as well as visceral (cf. Jansen and Brodal 1954, Daw and Moruzzi 1958, Newman and Paul 1966a, b, 1969, Evarts and Thach 1969, Oscarsson 1973).

The climbing fibres are axons from neurons in the inferior olive and exert a powerful excitatory influence directly on the Purkinje cells. The mossy fibres, having connections both from higher and lower CNS levels, make excitatory contacts with the granule cells. Each of these latter neurons excites via their axons the parallel fibres, around 45 Purkinje cells (Eccles 1973). The parallel fibres excite also the Golgi-stellate and basket cells which are all inhibitory in nature. The former type of interneurons exert an inhibitory action on the granule cells thus completing a negative feedback loop. The stellate and basket cells exert their inhibitory action on the Purkinje cells, and in such an arrangement as to focus the incoming excitatory discharge on a selected number of Purkinje cells. As a result of a steady stream of afferent impulses there is also during resting conditions a continuous activity in the Purkinje cells of the cortex as well as in the cells of the cerebellar nuclei which are as mentioned excited by collaterals from the climbing and mossy fibres.

The only output from the cerebellar cortex is mediated by the Purkinje cell axons which convey a uniformly inhibitory influence on the cerebellar nuclei and on some of the vestibular nuclei which functionally may be regarded as cerebellar. Collaterals from these axons also make contact with the Golgi and the basket cells. The Purkinje cells of the medial or vermal zone of the cortex project mainly on the fastigial nuclei and also on the vestibular nuclei while the paravermal cortex projects on the interposita nuclei (in man corresponding to the emboliform and globosa nuclei) and some vestibular nuclei. Lastly the Purkinje cells of the lateral hemispheric (neocerebellar) zones project upon the laterally placed dentate nuclei.

Thus the efferent connections from the cerebellum to other parts of the nervous system emanate from these different sets of cerebellar nuclei which in turn are exposed to a cortical inhibition as outlined above.

In this context the fastigial nuclei and their efferent pathways are of particular interest since the great majority of the cerebellar autonomic influences seems to be conveyed by these connections. The fastigial axons pass via direct fastigio-bulbar pathways or via the uncinate fasciculus to their main targets, the pontine and medullary reticular formation and the vestibular nuclei. In addition they also establish ascending connections to thalamic nuclei (Thomas et al. 1956) and to structures in the limbic system (Heath and Harper 1974).

By such general arrangements, and by receiving and modulating a wealth of information from various parts of the central nervous system and from peripheral receptors, the mentioned efferent connections efficiently modify outgoing motor commands into well coordinated motility patterns. Whether similar principles of cerebellar coordination are engaged also in autonomic control is still largely unknown but seems a priori likely. The first step to reveal such mechanisms is to explore whether autonomic effects

can be induced by stimulations of cerebellar nuclei or/and the corresponding cortical sections. From this type of experiments a considerable bulk of evidence is available as will be outlined below. A further step would be to investigate in more detail the patterns of autonomically mediated responses thus induced and to identify the efferent links and effector systems involved. The present studies have in general been organized along such principles and may help to elucidate the exact mode of influence exerted by the cerebellum on the visceromotor and hormonal control systems.

PREVIOUS STUDIES OF CEREBELLAR AUTONOMIC INFLUENCES

Cerebellar cardiovascular influences

Pioneer investigations of cerebellar influences on vegetative functions were performed already in the 19th century. Thus, in 1871 Owsjannikow reported that partial or total cerebellectomy in cats and rabbits had no influence on the blood pressure, while the following year Eckhard observed an increase in heart rate and blood pressure following stimulation of posterior vermal sections in dogs. Considerably later, Dresel and Lewy (1924) confirmed that vermal stimulation in dogs and rabbits led to a blood pressure increase and there were also other scattered observations at this time concerning cerebellar influences on cardiovascular mechanisms as surveyed by Wiggers (1943a) and Dow and Moruzzi (1958). Although certain discrepancies in results were obtained in these early exploratory studies, there were some general trends which can be summarized as follows: Electrical stimulation of the cerebellar cortex could modify heart rate and blood pressure in both directions, whereas cerebellar lesions did not appreciably change resting blood pressure. More precise interpretations of these early results can hardly be made, mainly because stimulation techniques were at that time poorly developed, with considerable risks for current spread.

Well controlled investigations on cerebellar autonomic control, especially concerning cerebellar influences on cardiovascular reflexes, were performed by Moruzzi (1938, 1940, 1947, 1950). Moruzzi found that stimulation of the anterior cerebellar lobe could markedly inhibit vasomotor reflexes while it hardly changed resting blood pressure. Thus, depressor responses elicited from afferent vagal fibres were inhibited by concomitant cerebellar stimulation. This was the case also with pressor responses whether induced by afferent sensory nerve stimulations or by bilateral carotid occlusion. This latter finding was confirmed by e.g. Wiggers (1943b), Hoffer (1965) and Hoffer, Ratcheson and Snider (1966). Relevant in this context are also observations by Smith and Nathan (1966) showing that stimulation of the inferior olive, which by way of the climbing fibres projects on the cerebellum, could inhibit the depressor component of the carotid sinus reflex.

Fitting into this pattern of cerebellar influences on vasomotor reflexes are reports by Reis and Cuénod (1965) that cerebellectomy enhanced the carotid baroreceptor reflexes in decerebrated cats. Vasomotor reflexes elicited by peripheral nerve stimulations were also affected by cerebellectomy, which may change pressor responses into depressor ones (Ranu and Bergmann 1967). On the other hand, cerebellectomy was reported to influence resting regional blood flow distribution only slightly and significant effects were confined to a reduction of blood flow to red skeletal muscle (Sheridan and Reis 1972). Also cerebellar stimulations, both in the cortex (Hoffer 1965) and in the fastigial nucleus (Mitra and Snider 1972) have been found to directly affect muscle blood flow.

As evident from the abovementioned studies, the impact of cerebellar cortical stimulations on the cardiovascular system is quite variable. Besides the importance of background vasomotor activity, the influence of anaesthesia on the response pattern has also been emphasized. Thus, circulatory

responses following cerebellar cortical stimulation have been reported to be markedly depressed or even reversed following barbiturate administration (Hoffer 1965 Hoffer et al 1966 Rasheed Manchanda and Anand 1970) Also the stimulation frequency used is of importance for the cerebellar cardiovascular responses (Hoffer 1965) Perhaps species differences are to be considered as well Inasmuch as rabbits seem to be more prone to display pressor responses while more variable blood pressure responses have been reported in cats

In the majority of the mentioned investigations the cardiovascular influences were induced from the cerebellar cortex, especially from the vermal part However In recent years the interest has been directed to the cerebellar nuclei where the fastigial nucleus is of particular interest since it receives most of its Purkinje cell axons from the vermal cortex Already in 1954 Zanchetti and Zoccolini observed that the fastigial nucleus was potent in producing pressor responses as part of a sham rage pattern In thalamic cats Later it was shown that a considerable well reproducible pressor response could be induced in intact cats from a small area confined to the rostral fastigial pole (Achari and Downman 1969 1970 Miura and Reis 1969 1970) This pressor response also present after intercollicular decerebration and characterized by widespread sympathetic activation (Achari and Downman 1970) as well as by an inhibition of vagal discharge to the heart (Lisander and Martner 1971b Achari Al-Ubaidy and Downman 1973) seems to be in some way related to the baroreceptor reflexes Thus Miura and Reis (1971) have described a site the paramedian reticular nucleus where an interaction between projections from the fastigial nucleus and the carotid sinus nerves seems to take place and also Nathan (1972) has presented evidence for fastigial projections on bulb structures engaged in vasomotor control Further the cardiovascular response pattern elicited by fastigial stimulation was closely similar to that evoked by baroreceptor unloading (Lisander and Martner 1971b Doba and Reis 1972a) and fastigial stimulation inhibits reflexly induced bradycardia whether produced by drugs like noradrenaline or phenyl diguanide (Achari et al 1973) by afferent sinus or vagal nerve stimulation (Hockman Livingston and Telenik 1970 Gurevitch and Vyshatina 1973) or by carotid sinus distension (Lisander and Martner 1971b) Even the release of ADH as elicited by baroreceptor unloading was suppressed by fastigial stimulation (Hata and Miura 1974) Concerning the possible functional significance of these cerebellar influences Doba and Reis (1972a 1972b 1974) have suggested that the fastigial nucleus might participate in orthostatic circulatory reflex adjustments since the cardiovascular responses to tilting were impaired following bilateral fastigial lesions in cats

With respect to cerebellar influences on autonomic mechanisms associated with particular behavioural adjustments Moruzzi observed already in 1947 that cerebellar stimulation in acute thalamic cats could suppress their spontaneously elicited sham rage responses i.e. interfere with the hypothalamically integrated defence reaction and its characteristic somatomotor and autonomic adjustments Such sham rage responses could also appear as rebound phenomena at the cessation of cerebellar cortical stimulation or be precipitated by fastigial stimulations (Zanchetti and Zoccolini 1954) Further evidence for a cerebellar hypothalamic interaction was presented

by Ban et al (1956) who reported that both hypothalamic pressor and depressor responses were abolished by concomitant stimulation of the anterior cerebellar lobe. In line with these reports Lisander and Martner (1971a) found that cortical stimulation of the anterior cerebellar lobe suppressed the autonomic components of the hypothalamic defence reaction including the cholinergic muscle vasodilatation.

These observations together with the findings by Zanchetti and Zoccolini that fastigial stimulation can precipitate sham rage raise the question in which way the fastigial projection influence the hypothalamic defence reaction directly or via descending relay stations. Further stimulation of the fastigial nuclei and adjacent cerebellar structures e.g. the lingula has been found also to induce oral behaviour in awake cats (Koella 1955, Reis, Daba and Nathan 1973, Bernthson, Potollecchio and Miller 1973) and rats (Baill, Mico and Bernthson 1974) suggesting that a variety of behavioural and autonomic patterns controlled by the limbic system and the hypothalamus might be modified by cerebellar mechanisms. Therefore one of the purposes of the present study was to explore whether and in which ways the cerebellum may affect autonomic neuroeffector systems other than the cardiovascular apparatus such as the gastrointestinal tract and the bladder and whether these autonomic adjustments are closely associated with particular behavioural changes or/and with cardiovascular adjustments. A preliminary report covering part of the present results has been published (Lisander and Martner 1974).

Cerebellar Influences on gastrointestinal function

Most of the relatively few reports dealing with cerebellar gastrointestinal influences have been concerned with the effects of cerebellectomy where constipation seems to be the most common finding (for ref see Dow and Moruzzi 1958). According to Bard et al (1947) removal of certain cerebellar parts including the nodulus, eliminate motion sickness dogs thus affecting also the autonomic gastrointestinal expressions.

In (1938) noted that cerebellectomy temporarily modified duodenal motility into a pattern of alternating periods of inactivity and excitation and Wolfe (1969) reported that restricted cerebellar lesions may produce gastric ulcerations.

Concerning effects of cerebellar stimulation Voronin and Zinkina (1938) observed longlasting inhibitions of small intestinal motility when the vermis and cerebellar hemispheres of cats were stimulated. Ban et al (1956) reported that gastric motility can be inhibited from the anterior cerebellar lobe in rabbits while both increased and decreased duodenal (Beller and Talan 1971) and gastric motility (Manchanda, Tandon and Anja 1972) have been reported in cats following cortical and fastigial stimulations. However no attempts appear to have been made to explore the pathways and peripheral mechanisms involved in the cerebellar modulations of gastrointestinal control.

Cerebellar effects on bladder function

Several investigations have reported cerebellar influences on micturition but with somewhat conflicting results particularly in earlier ablation experiments (see Dow and Moruzzi 1958). Stimulation of the cerebellar cortex can elicit both increases and decreases of bladder tone in intact cats (e.g. Whiteside and Guyton 1952; Emerson et al. 1961; Rasheed et al. 1970) as well as in decorticate animals (Bruhn et al. 1961). Chambers (1947) reported micturition upon stimulation of the fastigial nucleus and adjacent cerebellar structures while fastigial stimulations could inhibit the micturition reflex according to Bradley and Teague (1969). Both increases and decreases of bladder tone as elicited from the fastigial nucleus were reported by Beller and Talan (1971) and by Manchanda and Bhattarai (1972). The latter authors also noted that the responses although persisting were markedly changed after intercollicular brain stem section. Again however there appears to exist no detailed study where the cerebellar effects have been related to the peripheral pathways and neuroeffector mechanisms engaged in bladder control.

Miscellaneous cerebellar effects on autonomic hormonal control mechanisms

One of the oldest observations concerning cerebellar influences on autonomic and hormonal control systems were made by Claude Bernard in 1858 who reported hyperglycemia as a result of cerebellar lesion. Since then many studies with often contradictory results have been concerned with the role of the cerebellum in glucose regulation. At least part of the background for these effects may be a cerebellar influence on the sympathetic control of catecholamine release from the adrenal glands (Wada, Seo and Abe 1935) but also other types of mechanisms may be involved. Thus Kaplan (1938) reported changes in the glucose tolerance test lasting for up to 3 months following cerebellar removal. There are also scattered reports concerning cerebellar influences on such autonomic and hormonally mediated mechanisms as those governing thermoregulation and sexual function (for ref. see Wiggers 1943a; Dow and Moruzzi 1958).

It is clear from this brief survey concerning the role of the cerebellum in the regulation of autonomic and hormonal control systems that very little is known about the precise mechanisms involved. Thus most results hitherto are of an exploratory nature mostly indicating that a cerebellar influence appears to exist but only seldom offering any information about involved pathways, reflex arcs and levels of central integration.

AIM OF THE PRESENT STUDY

Although many investigations have thus demonstrated autonomic and even hormonal effects from the cerebellum very little is known about by which ways such influences are exerted. In the present series of investigations an analysis has been performed concerning the cerebellar influence on a number of defined autonomic functions which have earlier been extensively studied in this laboratory concerning other aspects of their central and reflex control. The interest was concentrated on the cerebellar fastigial nucleus since most of the cerebellar autonomic influences seem to be conveyed via this nucleus and the cortical region the vermis which projects on its neurons.

In the first paper (I) the interrelation between the fastigial nucleus and the hypothalamic defence area was investigated as an extension of an earlier study which showed that the hypothalamic defence reaction could be suppressed from vermal parts of the cerebellar anterior lobe (Lisander and Martner 1971a). Since the Purkinje cell axons from this cortical area exert an inhibitory influence on the fastigial neurons (see earlier sections) it was considered of interest to explore the fastigial influence on the defence reaction.

In the subsequent paper (II) the question was raised whether and in which ways the fastigial nucleus besides the cardiovascular influences induced from the fastigial pressor area conveys effects also on other autonomically innervated organ systems. The interest was here concentrated upon effects on intestinal motility as elicited by fastigial stimulations with an analysis of the autonomic pathways involved.

Extended information concerning the organization of cerebellar autonomic control might emerge from investigations of cerebellar influences on such functions that involve both somatomotor and autonomic mechanisms. For such reasons the defecation mechanism was chosen having the additional advantage that possible cerebellar influences on autonomic response patterns conveyed by the sacral parasympathetic outflow could be studied. This is the case also for the micturition reflex and paper III was therefore devoted to an analysis of the cerebellar influence on the defecation and micturition mechanisms.

The extrinsic nervous control of gastric motility is particularly well developed involving besides adrenergic inhibitory and cholinergic excitatory mechanisms also the vagal relaxatory fibres. Utilizing a so far unknown transmitter these latter fibres control the reservoir function of the stomach (Marlinson 1965). The nervous control of the stomach therefore allows for highly differentiated response patterns via the mentioned three efferent autonomic links and may if modified from the fastigial nucleus offer interesting information about the organization of cerebellar autonomic influences (paper IV).

In any study of autonomic function control of the background reflex activity is a prerequisite for a detailed analysis of the mechanisms involved and this is certainly true also when superimposed cerebellar control mechanisms are investigated. Abdominal surgery profoundly interferes with gastrointestinal background tone mainly by adding reflex inhibitory effects.

Induced by peritoneal irritation Intestinal handling etc Since these inhibitory reflexes appear to be conveyed mainly via the adrenergic sympathetic supply to the gastrointestinal tract (see Furness and Costa 1974) the cerebellar influence on neurogenic intestinal control was explored both before and after such reflex mechanisms were superimposed by inflicting abdominal trauma or irritation These aspects were explored both in paper IV and V

Lastly further indications of the functional importance of the fastigial nucleus concerning autonomic control might be obtained from studies where this cerebellar part is stimulated in unanaesthetized animals with chronic indwelling electrodes and such experiments are described in the last paper (V) This technique has also the advantage of avoiding anaesthesia which in itself may alter cerebellar responses In these cats it was possible to study behavioural responses to fastigial stimulation in relation to simultaneously induced cardiovascular and gastrointestinal adjustments

METHODOLOGICAL CONSIDERATIONS

For a detailed description of methods the different papers should be consulted and only a general survey will be given below. The experiments were performed on altogether 150 cats. In the acute experiments chloralose was used as anaesthetic. Cerebellar autonomic influences were evaluated by recording autonomic effector responses e.g. heart rate, blood pressure, regional blood flow, gastrointestinal and bladder motility etc. This type of recording was judged to serve the present purpose better than electrophysiological recordings which may give results difficult to translate into functional patterns. For example as will be illustrated below, fastigial stimulation enhances the sympathetic excitatory discharge to blood vessels while it suppresses prevailing activity in the sympathetic fibres that inhibit intestinal motility. Thus if cerebellar effects were analysed only by recording changes in spike activity in e.g. the splanchnic nerves it would be very difficult if not impossible to interpret the discharge pattern obtained in terms of neurogenic effects on the cardiovascular and gastrointestinal effectors. The net effect on electrical activity as recorded in splanchnic nerve bundles may then even appear to be largely unchanged despite pronounced cerebellar influences on both gastrointestinal motility and blood flow since the mentioned decrease and increase respectively in adrenergic discharge mediating these effects might more or less cancel out. It would further be impossible to differentiate between vasodilator and vasoconstrictor fibres but in terms of elicited effector responses they mean the very opposite to each other.

Stimulations

For central stimulations both in the hypothalamus and cerebellum the Horsley-Clarke technique was utilized. Stimulation was performed with monopolar steel electrodes using constant current usually between 0.05-0.2

The insulated tips to the electrodes were about 100 μ m in diameter. Stimulation of central nervous mechanisms using topical electric stimulations is always at hand. In the present study the danger of undue current spread is always at hand. In the present study the problem had to be particularly carefully considered because of the close proximity between the fastigial nucleus and the brainstem. However the following factors strongly indicate that the obtained responses to topical stimulations were true fastigial effects and not due to current spread to bulbar structures. First the responsive area was quite small and minor shifts (0.5 mm) in electrode position even towards the brainstem lead to a disappearance of the stimulation effects. Second the responses also disappeared if a small tissue lesion was applied around the electrode tip. Third histological examination confirmed that the stimulation sites were located in or close to the fastigial nucleus (see below). Fourth threshold intensities were often quite low and values below 0.05 mA were sometimes sufficient to elicit responses.

In the cats used for acute experiments a tracheal cannula was inserted to secure free airways and also to allow for artificial respiration. To eliminate catecholamine release from the adrenals these glands were ligated in cats subject to laparotomy and hydrocortisone substitution was then given.

A The cardiovascular system Arterial catheters allowed for recordings of heart rate and blood pressure. In the chronic awake animals such registrations could be obtained by means of an indwelling aortic catheter inserted via the right carotid artery. Blood flow was recorded as the venous outflow from the organ under study. The neurogenic control of muscle blood flow was sometimes estimated by using the cross circulation technique. The isolated calf preparation with intact nervous connections was then perfused with blood from a donor cat thus allowing for flow recordings that were undisturbed by experimentally induced pressure variations in the recipient (paper I).

B The gastrointestinal tract Gastric motor responses were recorded as isometric volume changes in a large waterfilled intragastric balloon. This method reflects net changes in gastric volume rather than individual contraction waves and has the advantage of avoiding variations in gastric pressure that might per se induce gastric motor reflexes (for details see Jansson 1969a). The intragastric balloon was inserted via the esophagus in the acute experiments (paper IV) and via an earlier made gastric fistula in the chronic cats (paper V). Both methods had the advantage of not requiring laparotomy.

Intestinal motility was in most cases recorded as volume changes in isolated intestinal segments which via a wide tube were connected to a reservoir containing Tyrode solution. By changing the level of the container the intraluminal pressure could be adjusted to induce a desirable level of intrinsic intestinal motor activity against which e.g. the adrenergic impact on motility could be tested. Like the situation in the stomach this kind of recording generally reflects overall changes in tone in the intestinal segment and is therefore more suitable for the present purpose than identification and classification of individual contraction waves. Further the danger of mistaking respiratory movements for intestinal peristalsis will be reduced.

In addition the following precautions were undertaken to differentiate gastrointestinal motor action from somatomotor effects. 1 Neuromuscular blocking agents were often used and artificial respiration given. 2 In the laparotomized cats direct inspections of the stomach and intestine confirmed that gastrointestinal motor activity corresponded to the recorded changes in motility. 3 When intestinal motility was recorded the intestinal segment under study could be brought entirely outside the abdomen thus abolishing every possible somatomotor influence on the recordings.

The above mentioned method of recording intestinal motility required laparotomy. To avoid acute abdominal surgery which profoundly influences intestinal motility and reflexes a segment of the small intestine was iso-

lated in one group of cats at an operation 1-2 weeks prior to the acute experiment (paper V). The ends of this segment emerged through the abdominal wall to form a so-called Thiry Vella loop. In which motility can be recorded without inflicting any acute trauma. In all cats with Thiry-Vella loops (paper V) and in a few of the acute experiments (paper II) intestinal motility was recorded as pressure changes in intraluminal balloons.

The somatomotor component of the defecation reflex was registered as pressure changes in an intraabdominally placed balloon, thereby reflecting straining movements which could also be easily observed by direct inspection (paper III).

C The bladder Bladder motility was recorded as intravesical pressure changes by an urethral catheter inserted transabdominally (paper III). Via a side branch the bladder could be filled with warm saline at different pressures. The pressure level was very important for the outcome of the responses.

Analysis of mechanisms

A Efferent pathways The efferent mechanisms mediating the cerebellar effects were analyzed by a combined use of anticholinergic or anticholinergic drugs and nerve sectioning, or by reversible cold blockade of peripheral nerves. The nerves to be cut or reversibly blocked in the course of the experiment were dissected free with great caution.

B Background reflex control Since modulation of autonomic reflex activity seems to be one important mechanism for cerebellar autonomic interaction, it was necessary to maintain proper control of the activity in reflexes impinging on the organ under study. Increased activity in these autonomic reflex arcs could be induced by various means, e.g. by electrical mechanical or chemical stimulation of the respective afferent fibres. Hence the vago-vagal relaxatory reflex was elicited by graded afferent stimulation of one vagal nerve while the other nerve was left intact for conveying the efferent discharge (cf. Jansson 1969b). The defecation reflex was elicited either by applying a stimulation of the cut pelvic nerves on one side, or by mechanical stimulation of the rectal mucosa (cf. Hultén 1969). These methods of initiating defecation complemented each other inasmuch as the colonic motor and blood flow adjustments induced by the adequate mechanical stimulus could also be evoked by afferent pelvic nerve stimulation revealing that these responses were not due simply to any intramural reflex or to mechanical artifact.

The Testino-intestinal (gastric) inhibitory reflex, which proved to be of particular relevance for cerebellar gastrointestinal control, could be initiated by several different stimuli. First, laparotomy inevitably induced long lasting neurogenic inhibitions of gastrointestinal motility by reflexes which could be reinforced by afferent stimulation of mesenteric nerves or by distension of an intestinal segment. In the intact animals these reflex inhibitions could be reversibly elicited by intraabdominal injection of small amounts of irritating fluid, such as acids or alkali. The cats with Thiry-

Vella loops (paper V) were particularly suitable for studying the Intestino-Intestinal (gastric) inhibitory reflex since graded intestinal distensions could here be performed without laparotomy

Histological methods

Identification of the electrode tracks and stimulation sites was performed by histological examination of the cerebellum. In papers II-V the stimulation sites giving rise to cerebellar autonomic responses were plotted on maps of the relevant cerebellar structures. It must however be realized that it is not possible to localize the stimulated area with greater accuracy than about 1 mm. Thus, if data obtained from the hypothalamus (Wise 1972) can be applied also on the cerebellum, the current spread with the present stimulation parameters might be up to 0.5 mm. Another 0.5 mm error comes from the size of the electrolytic lesions made to indicate the stimulation sites.

RESULTS AND COMMENTS

Influences on the cardiovascular defence reaction (paper I)

Stimulation of the hypothalamic defence area induces a widespread but differentiated sympathetic discharge to the circulatory system causing blood pressure rise tachycardia increased vascular resistance in most vascular beds except in skeletal muscle and an adrenal catecholamine release. In skeletal muscle an increased blood flow is instead induced due to activation of cholinergic vasodilator fibres and to the vasodilator effect of blood borne adrenaline. As a continuation of an earlier study showing that the cerebellar cortex can suppress the autonomic components of the defence reaction (Lüscher and Martner 1971a) the fastigial influence on these cardiovascular adjustments was investigated and special attention was paid to the changes in muscle blood flow.

Fastigial stimulation which in itself produces an adrenergically mediated pressor response and a decreased muscle blood flow was found to suppress the hypothalamically induced cholinergic vasodilatation in the skeletal muscles whereas the adrenergically mediated components of the defence reaction were potentiated. The fastigial inhibition of the neurogenic muscle vasodilatation might a priori be due to either a central suppression of the vasodilator fibre discharge or to an increased constrictor fibre discharge which hemodynamically interferes with the vasodilator fibre influences. The latter possibility is a realistic one since the effects of the vasodilator fibres may be nearly abolished by a concomitant vasoconstrictor fibre activity (Folkow Öberg and Rubinstein 1964) and such vasoconstrictor fibre activity is indeed induced by stimulation in the fastigial pressor area.

Therefore special arrangements had to be undertaken to investigate whether the fastigial stimulation also caused a suppression of vasodilator fibre activity per se. Blood flow was therefore recorded in a cross-circulated, adrenergically blocked calf muscle preparation (see Methods) which was perfused at constant pressure from a donor cat. In this way the impact of cholinergic vasodilator fibres could be studied without any interference by the vasoconstrictor fibres. Even then the muscle blood flow increase during defence area stimulation could be suppressed by fastigial stimulation suggesting that the fastigial neurons interfere centrally with the activation of the sympathetic cholinergic vasodilator fibres. However whether this interaction occurs at the hypothalamic level or at lower levels of the neuraxis is unclear. Fastigial stimulation alone did not influence blood flow in the adrenergically blocked muscle vascular bed indicating that the cholinergic vasodilator fibres are not activated by stimulation of the fastigial neurons.

In some cats treated with the adrenergic blocker guanethidine and hence deprived of the vasoconstrictor fibre influence fastigial stimulation could potentiate a delayed muscle vasodilator response induced by defence area stimulation. This potentiated dilator response characterized by long latency and duration was unaffected by atropine but blocked by β -blocking agents. When taken together these observations indicate that the fastigial stimulation potentiated the adrenaline release from the adrenal medulla.

known to be induced by defence area stimulation (cf Grant et al 1958) Guanethidine does not block the hormonal component of the sympatho-adrenal system but rather tends to potentiate the peripheral effects of adrenaline (Abercrombie and Davies 1963)

A fastigial facilitation also seems to take place regarding the adrenergic vasoconstrictor fibre discharge to the intestinal cutaneous and muscle vascular beds as elicited by defence area stimulations. This was evident from experiments where the vasoconstrictor fibre influence could be recorded without interference from circulating catecholamines: changes in perfusion pressure or from baroreceptor reflex modifications. Such arrangements are necessary prerequisites for detecting the slight adrenergic vasoconstrictor fibre activation to the muscle vascular bed which can be elicited from the defence area as long as the baroreceptor reflexes are hindered from changing this response into one of vasoconstrictor fibre inhibition (cf Lüscher 1971)

In conclusion the hypothesis that the cerebellar cortex and nuclei influenced the defence reaction in mutually throughout opposite directions could not be verified. Insofar as the fastigial nucleus exerted a facilitatory influence on the adrenergically mediated links but suppressed the cholinergic vasodilator fibre influence.

These findings appear to fit well with reports by Achari et al (1973) who observed a mutual fastigial hypothalamic facilitation concerning the adrenergic but not of the behavioural components of the defence reaction. At first sight it is somewhat surprising that fastigial stimulation may suppress part of the defence reaction i.e. the cholinergic vasodilatation since such stimulations can induce sham rage in decorticate cats (Zanchetti and Zoccolini 1954). However the situation is different in fully anesthetized cats compared to that of decorticate animals where sham rage can be triggered by very slight stimuli of unspecific trivial nature or may even occur spontaneously (Bard 1928). These results will be discussed more in detail later on.

Influences on Intestinal motility

Effects in acutely laparotomized cats (paper II) In this study it was explored whether intestinal motility is influenced by fastigial stimulation besides the mentioned effects on the autonomic control of the cardiovascular system. Recordings of motility were performed in the jejunum, ileum and colon and fastigial stimulation was found to influence motility in all these intestinal parts though in a differentiated way. Thus the jejunum responded with both excitatory and inhibitory patterns whereas the ileum uniformly responded with increased motor activity. Also the colon displayed an excitatory pattern with a tendency for the proximal parts to be more responsive than distal segments.

Analysis of the autonomic mechanisms which conveyed the intestinal responses was performed by the use of different blocking drugs in combination with sectioning or cold blockade of the peripheral autonomic nerve supply. It was then found that interruption of the parasympathetic pathways the vagi for the small intestine and for the colon in addition the pelvic

that there is little activity to be inhibited

Effects in cats with Thiry Vella loops (paper V) To further elucidate the fastigial influence on intestinal motility and especially its relation to the Intestino-Intestinal Inhibitory reflex arrangements were made to record motility atraumatically (see Methods) thus avoiding the elicitation of inhibitory reflexes. In such a situation it is likely that the effects of fastigial stimulation on intestinal motility are different. As shown in Fig. 1 panel A ileal motility is now depressed by fastigial stimulation which was never seen when motility was recorded in laparotomized cats (paper II). Panel B reflects the situation after vagotomy illustrating that the extrinsic parasympathetic pathways are not necessary for eliciting the ileal inhibitory responses. Subsequent laparotomy depresses the spontaneous ileal motility and the same fastigial stimulation which before laparotomy induced motility inhibition (panel A) now enhances intestinal motility (panel C). Evidently the initial inhibitory effects by fastigial stimulation are not conveyed via the vagal parasympathetic supply which means that they must be due to an activation of the adrenergic inhibitory supply suppressing the prevailing activity in intramural cholinergic neurons. However once a reflex activation of adrenergic inhibitory discharge is induced by abdominal trauma, the same fastigial stimulation tends to suppress this prevailing activity. In other words it appears as if the fastigial neurons enhance the adrenergic fibre activity to the intestine if initially low or absent but suppress it when it is initially marked. In contrast concerning the adrenergic fibre control of the cardiovascular system only excitatory influences from the fastigial nucleus have been observed.

Influences on defecation and micturition (paper III)

Since the fastigial nucleus evidently is involved in adrenergic regulation of both cardiovascular (paper I) and intestinal functions (paper II) it is considered of interest to investigate if also parasympathetically conveyed autonomic patterns are influenced. The micturition and defecation reflexes were chosen as examples of such autonomic mechanisms. Furthermore the latter reflex also serves as an example of a pattern in which both autonomic and somatomotor components are closely interwoven and might for this reason be of particular interest for analyses of the mode of cerebellar modulation.

Defecation Mechanical rectal stimulation or afferent pelvic nerve stimulation elicits a reflex increase in colonic blood flow and motility as well as straining movements (cf. Hultén 1969). All these adjustments associated with defecation were found to be influenced by fastigial stimulation. Thus both the colonic motor response and the increased blood flow were reduced or totally inhibited while the straining movements were always entirely abolished by a concomitant fastigial stimulation. In no case were any of these components of the defecation reflex enhanced by fastigial stimulation. The stimulation sites influencing the defecation reflex were located in the rostral fastigial pole.

In all these experiments adrenergic blockade was used to eliminate the above described (paper II) fastigial modulation of colonic motility via the adrenergic fibre supply which might interfere with the recordings of parasympathetically mediated responses. This procedure was also necessary for obtaining a full expression of the parasympathetic colonic motility and vascular effects which are known to be suppressed by a prevailing activity in the sympathetic fibre supply (e.g. Hultén 1969). The results indicate that the fastigial neurons can markedly suppress all the components of the defecation reflex but at which level of the neuraxis this influence is exerted is unclear. It is certainly not at the receptor level, e.g. by some type of neurogenic suppression of intramural mechanoreceptor sensitivity since the fastigial suppression of the defecation reflex was the same whether the reflex was induced by adequate mechanical stimulation of the rectal wall or by afferent pelvic nerve stimulation. Spinal as well as bulbar and suprabulbar centres seem to be involved in defecation (cf. Koppányi 1930, Hess and Brügger 1943, Hultén 1969) and the cerebellar interaction might occur at any of these levels.

Micturition. Fastigial stimulation was found to cause either facilitation or inhibition of bladder tone. The background bladder tone as well as the electrode position in the fastigial nucleus could both affect the direction of the response. Thus a reversal from excitation to inhibition of bladder tone could be observed upon even slight changes in electrode position. Further it was sometimes possible to induce both increased and decreased motility from the same stimulation site depending on the initial state of the bladder. Thus if bladder tone was initially high, suppression of this parasympathetically mediated tone was likely to ensue; but the same fastigial stimulation could induce bladder contraction if started during a period of bladder inactivity. Such a dependence on background reflex activity concerning bladder responses has been reported also in experiments where the cerebellar cortex was stimulated (Whiteside and Guyton 1952). The suppression of bladder tone as a result of fastigial stimulation was not due to any sympathetic activation since it was unaffected by complete blockade of adrenergic fibres. Therefore the fastigial inhibitory influence on the micturition reflex must be ascribed to a central suppression of parasympathetic activity as also suggested by Bradely and Teague (1969).

The fastigial structures affecting the parasympathetic bladder control had a larger extent than those from which pressor and intestinal motility responses were elicited. Further it was not possible to distinguish distinct areas from which excitatory and inhibitory bladder effects could be elicited. Not seldom rebound bladder excitations were observed upon cessation of fastigial stimulations and rebound effects have been observed also in connection with sham rage responses (Moruzzi 1947, Zanchetti and Zoccolini 1954).

Influences on gastric motor function (paper IV)

This study was undertaken to investigate possible fastigial influences on the particularly complex autonomic control of the stomach. Since this organ is supplied with two sets of vagal fibres the excitatory and relaxa-

tory ones and also with sympathetic inhibitory fibres. It provides an interesting model for testing cerebellar influences on various types of autonomic control. The stomach also offers the advantage that the motility control can be conveniently studied without laparotomy or other surgical interferences that tend to elicit sympathetically mediated inhibitory gastric reflexes. Moreover background cholinergic activity in the stomach can be easily controlled by graded efferent vagal stimulations and also graded vago-vagal relaxatory reflexes can be elicited.

The results of the present study indicate that the fastigial nucleus can influence gastric motility in a complex way by evoking both suppression and facilitation (Fig. 1). If however the prevailing experimental conditions were under strict control and properly considered when evaluating the results, it proved possible to analyse the main mechanisms involved. Theoretically changes in gastric motor function might be induced by discharge variations in the inhibitory adrenergic or excitatory cholinergic nervous pathways as well as via altered activity in the vagal relaxatory fibres and also hormonal mechanisms might be involved. All these possibilities were taken into account when the fastigial effects on the stomach were explored.

In intact animals fastigial stimulation elicited excitatory as well as inhibitory responses sometimes combined to form biphasic patterns. After guanethidine the inhibitory gastric responses were either completely eliminated or sometimes only reduced but then with a changed and considerably delayed response pattern. These observations indicate that the effects were mediated by sympathetic nerves and also to some extent by adrenal catecholamine release. Parasympathetic blockade also interfered with these responses (Fig. 1 panel B) but as will be discussed below these observations do not necessarily imply that activity changes in such pathways are involved. Excitatory gastric responses to fastigial stimulation appeared at least in part to be caused by activation of the vagal cholinergic pathways since the effects persisted after high spinal cord transection but were abolished by atropine. These results illustrate how the fastigial neurons can also excite parasympathetic pathways. In this case the vagal excitatory effects to the stomach.

The vagal relaxatory fibres whose transmitter mechanism is unknown are reflexly activated by e.g. gastric distension and even the low pressures exerted by the present recording balloon is usually sufficient to elicit this reflex (cf. Abrahamson and Jonsson 1973). Both afferent and efferent pathways of this reflex run in the vagal nerves and it was found that fastigial stimulation suppressed this vago-vagal reflex thus displaying another way of cerebellar modulation of gastric tone. Moreover this interaction illustrates how the cerebellar control of autonomic mechanisms is not only confined to the adrenergic and cholinergic pathways.

In laparotomized animals a different type of motility responses was obtained. After acute abdominal surgery or intraperitoneal injection of irritating fluid procedures which elicit sympathetically mediated inhibitory reflexes to the stomach, fastigial stimulation always resulted in excitatory gastric responses independent of the direction of preceding stimulatory effects (Fig. 1 panels A and C). As seen from the figure the initially obtained gastric inhibitions were by laparotomy reversed into excitatory responses.

which were conveyed by adrenergic fibres and in all likelihood caused by fastigial suppression of adrenergic inhibitory reflexes to the stomach. These results therefore agree closely with those described above concerning intestinal responses in cats with Thiry-Vella loops where also reversed responses were obtained after laparotomy. If in animals with a Thiry-Vella loop a distending pressure was applied to this loop gastric motility was depressed via the intestino-gastric inhibitory reflex. This reflex inhibition of gastric motility could be suppressed by a concomitant fastigial stimulation even when such a stimulation per se induced a depression of gastric tone (paper V).

However, a prevailing cholinergic nervous activity whether intrinsic or extrinsic is a prerequisite for demonstrating the adrenergic inhibitory influence on gastrointestinal motility. This presumably reflects the fact that the influence of the sympathetic inhibitory fibres to the gastrointestinal tract is almost entirely confined to an action on the intramural cholinergic ganglia to judge from morphological (Norberg 1964) and functional studies (Kewenter 1965; Jansson and Martinsson 1966; Hultén 1969). Accordingly, no effects whatsoever of adrenergic discharge can be induced if no cholinergic activity is present (cf. Fig. 1, panels A and B). Whenever the cholinergic pathways were interrupted it was therefore necessary to induce such activity either via stimulation of the extrinsic parasympathetic nerves or by facilitating intramural ganglionic activity e.g. by exposing the intestine to a suitable distending pressure.

The fastigial interaction with sympathetically conveyed intestino-gastric inhibitory reflexes persisted following intercollicular decerebration; i.e. structures cranial to the midbrain are not essential for this type of cerebellar modulation (paper IV).

Evidently fastigial neurons can modulate the activity in all the three efferent sets of autonomic fibres that contribute to gastric motility control and these influences are furthermore exerted from the same restricted fastigial section i.e. the fastigial pressor area. Analysis of the frequency-response relationships for the fastigial effects revealed that those representing the cardiovascular and the gastric effects showed closely similar characteristics. Thus there may be close anatomical and functional relationships between the fastigial neuron pools that influence these types of autonomic mechanisms, possibly even the same fastigial neurons might be involved.

To summarize, the fastigial nucleus can induce inhibition of gastric motility by inducing an increased adrenergic discharge and/or an adrenal catecholamine release. Increased gastric motility can be effected by facilitation of vagal cholinergic discharge by inhibition of a prevailing activity in vagal relaxatory fibres and in situations of abdominal irritation or lesions, by suppression of adrenergic inhibitory reflexes. Which of these mechanisms that dominates the pattern of the response in a given situation depends largely upon the prevailing background of autonomic activity.

Behavioural and gastric responses in conscious cats (paper V)

To gain further information concerning the functional importance of the fastigial nucleus in autonomic control observations were made also in

unanaesthetized cats with indwelling fastigial electrodes. In these cats arrangements were made for registration of gastric motility and blood pressure without any acute surgical intervention thus allowing for simultaneous observations of circulatory, gastric and behavioural adjustments in response to fastigial stimulation. The idea was to explore whether any particular behavioural patterns were associated with the autonomic responses or might be secondarily induced as a result of such autonomic adjustments.

As in anaesthetized cats fastigial stimulation induced in the conscious freely moving animals pressor responses but in addition patterns of more or less obvious oral behaviours. In this respect confirming findings by Reis *et al* 1973 and Bernson *et al* 1973. The character of these behavioural responses was partly dependent on the stimulation intensity. Thus low current levels initiated sporadic licking of mouth and fur, an activity which became more vigorous with increasing intensity. At still higher intensities the grooming was replaced by biting and chewing and in one cat eating was induced. All these responses started and ended within few seconds after onset and cessation of stimulation and could be easily reproduced. The responses were furthermore performed in a well coordinated and stereotyped fashion and the animals showed no signs indicating that the stimulations might have been disagreeable. These specific somatomotor responses did not take the form of individual limb movements or other types of restricted motility patterns which often seems to be the case when other cerebellar nuclei are topically stimulated in conscious animals (e.g. Chambers 1947, Koella 1955).

In contrast to the pronounced effects on blood pressure and behaviour fastigial stimulation did not conspicuously influence gastric motor activity and only in a few cases were gastric inhibitions observed resembling the adrenergically mediated relaxations in anaesthetized cats (paper IV). Neither was the fastigially elicited oral behaviour associated with any measurable increase in gastric hydrochloric acid secretion. The absence of regularly appearing gastric responses in association with the oral behaviours indicates that the latter can hardly be secondary to fastigially induced gastric ad-

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In subsequent acute experiments during anaesthesia on the same animal fastigial stimulation elicited the earlier described inhibitory or bil gastric responses. After abdominal surgery these responses changed into uniformly excitatory effects indicating suppression of intestino-gastric inhibitory reflexes. This gastric response pattern was thus similar to that found in the anaesthetized cats reported in paper IV. Furthermore histological examination showed that it was elicited from the same cerebellar area i.e. from the rostral fastigial pole. As a matter of fact most autonomic influences (paper I V) and also the oral behaviours reported in paper V were elicited from this area. Thus the fastigial pressor area seems to form a site where many not necessarily interrelated kinds of autonomic and behavioural mechanisms may be modulated; observations which are in consonance with reports by Reis *et al* (1973).

GENERAL DISCUSSION

In recent years a considerable amount of information has accumulated confirming early observations indicating an involvement of the cerebellum also in functions beside its wellknown control of somatomotor mechanisms. For example cerebellar influences on autonomic regulation, especially concerning cardiovascular mechanisms, have been repeatedly demonstrated. Further, there are reports of behavioural and emotional effects following cerebellar lesions (Peters and Mojan 1971) and stimulations (Reis et al 1973, Bernston et al 1973, Ball et al 1974). The fastigial nucleus is of particular interest in this context since the great majority of the cerebellar autonomic adjustments reported in the literature have been ascribed to structures having their efferent pathways via this nucleus.

From the rostral fastigial pole powerful and well reproducible pressor responses can be elicited by electrical stimulation (Achari and Downman 1969, 1970, Miura and Reis 1969, 1970). The present results indicate that this pressor area, in addition to its cardiovascular influences, is involved also in several other aspects of autonomic regulation. For example colonic and small intestinal motility (paper II), defecation and micturition (paper III) and different reflex arcs involved in gastric motor control (paper IV) can be markedly influenced by fastigial stimulation. Thus both the para-sympathetic pathways, vagal and sacral, and the sympathetic inhibitory nerve supply can be modulated, as well as the hormonal link of the sympatho-adrenal system. This latter example of hormonal involvement is in agreement with recent demonstrations that fastigial stimulation can affect ADH secretion as well (Hata and Miura 1974). In addition oral behaviours can be elicited from the fastigial pressor area (paper V), confirming findings by Reis et al (1973).

It therefore appears as if a great variety of autonomic adjustments can be induced from a quite restricted section of the cerebellar efferent outflow, involving virtually all links of the parasympathetic and sympatho-adrenal systems and consequently their subordinated effector organs. However, these different autonomic effects, which can almost all be induced from the same electrode position in the fastigial nucleus, do not form the same type of regular and wellintegrated response patterns as those which can be elicited from e.g. the hypothalamic feeding area or defence area. While these latter responses are always the same in direction, it seems as if fastigial neurons exert rather a modulating influence on autonomic control mechanisms in general, often damping prevailing activity when this is high but exerting a facilitating influence when activity is initially low. Such a type of influence largely seems to hold for the gastrointestinal system, while the fastigial influence on sympathetic activity to the cardiovascular system appears to be mainly of a facilitatory nature. This is so even when the cardiovascular sympathetic discharge is enhanced by baroreceptor unloading (Achari and Downman 1970, Miura and Reis 1971, Lisander and Martner 1971b) or defence area stimulation (Achari et al 1973, paper I). This concentration of a variety of vegetative and hormonal influences to a very restricted part of the fastigial nucleus is in line with observations that the fastigial neurons seem to be aggregated in colonies of related performance (Eccles et al 1972).

due to a descending direct interaction with neurons or receptors outside the central nervous system even though the principle of e.g. efferent neurogenic modulation of receptor sensitivity is generally accepted. Such a view is furthermore supported by the following observations: First, fastigial stimulation was found to modulate the defecation reflex equally efficiently whether the reflex was induced by a physiological excitation of rectal receptors or by afferent pelvic nerve stimulation (paper III). Second, fastigial stimulation was found to influence all autonomic reflexes tested in this series of investigations with one notable exception. Mechanical stimulation of the intestinal mucosa elicits an intestinal vasodilatation which is fairly unique by being conveyed via strictly intramural reflex connections (see Biber 1973) and this type of extraspinal autonomic reflex was not influenced by fastigial stimulation (Martner unpublished observation).

Cerebellar influences on spinal or/and bulbar autonomic structures appear as probable levels for interaction. In fact, several autonomic reflexes known to be relayed on these levels are liable to cerebellar modulation. For example, the cerebellum can influence cardiovascular (Maruzzi 1938, 1940, Stella and Stevan 1962) micturition (Bradley and Teague 1969) and gastric reflexes (paper IV). Further, these mentioned modulations are all present in animals subject to decerebration at pre- or intercollicular levels showing that these particular cerebellar influences are not relayed at CNS centres cranial to mesencephalon. Also the fastigial pressor response does not require higher levels of the nervous system since decerebration does not abolish the blood pressure increase upon fastigial stimulation (Achari and Downman 1969, Miura and Reis 1969).

There are, however, indications that also higher autonomic centres like the hypothalamus and the limbic system may be exposed to cerebellar influences concerning the vegetative functions that are integrated at these levels. Thus, the cerebellum can influence ADH release (Hata and Miura 1974) and also induce sham rage (Zanchetti and Zoccolini 1954). Further cardiovascular and gastric responses elicited from the hypothalamus can be influenced by cerebellar stimulation (Ban et al. 1956) as is the case with autonomic components of the hypothalamic defence reaction (Lüscher

Martner 1971a, paper I). However, an interaction with relay centres ²⁴the ascending or descending pathways can of course not be excluded in these cases. Thus, the fibre system that controls the sympathetic cholinergic vasodilator fibres has synapses both in the mesencephalon and in the spinal cord (Lindgren 1955). Further indications of a cerebellar hypothalamic interrelation come from observations that some cardiovascular responses elicited by cerebellar stimulation are abolished by precollicular decerebration (Ban et al. 1956) or by diencephalic coagulation (Ban, Hilliard and Sawyer 1960, Sawyer, Hilliard and Ban 1961).

Even if the results from the studies mentioned above clearly demonstrate an interaction between limbic/hypothalamic influences and cerebellar influences, they cannot be taken as conclusive evidence for the assumption that some cerebellar effects are really relayed at hypothalamic and limbic levels. The mentioned interaction may well take place in centres at e.g. bulbar levels whose functions are affected by and dependent on both types of influences.

There is, however, neuro-anatomical evidence for connections between

the cerebellum and the hypothalamus or/and the limbic system. Such connections have been demonstrated both with electro-physiological methods (e.g. Ban and Inoue 1956, Anand et al 1959, Heath and Harper 1974) and with histological studies (Heath and Harper 1974).

Exactly how the fastigial induction of the oral behaviour is effectuated and which nervous structures that are involved is of considerable interest but is so far not known. In case the cerebellum is capable of learning and memorizing patterns of somatomotor performance as proposed e.g. by Marr (1969) and Gilbert (1974) fastigial stimulation might have activated learned sequences of movements involved in oral behaviour. It is however difficult to understand how such an admittedly crude interference with the delicate cerebellar machinery as a topical electrical stimulation implies can induce such complex and well coordinated motor performances. A more reasonable explanation seems to be that the fastigial neurons exert a general facilitating influence on centres that normally initiate and integrate oral behavioural patterns e.g. the hypothalamic feeding area, the limbic system etc. The amygdala might here be of particular interest since oral behaviour can be particularly easily induced by stimulation in this region (cf. Gloor 1960) and it was recently shown that the amygdala receive ascending projections from the fastigial nucleus (Heath and Harper 1974). Also a fastigial facilitation of bulbar relay centres might be of relevance for the oral responses since even rather complex motor performances can be integrated at these lower levels of the neuraxis (cf. Bard and Macht 1958). The incitement of oral behaviour by unpleasant sensations induced by cerebellar stimulation (Chambers 1947, Koella 1955) is a third possibility which however appears unlikely since self-stimulation can be obtained from the same areas that produce these response patterns (Ball et al 1974).

Importance of background reflex activity

As mentioned above it is most likely that the cerebellum exerts its autonomic influence by modifying reflex relay stations and higher centres controlling autonomic neuroeffectors. If so the prevailing reflex activity must be of most importance for determining the response to cerebellar stimulation which also appears to be the case. Thus cardiovascular (Moruzzi 1938, 1940), gastrointestinal (Monchanda et al 1972, paper II, IV and V) and bladder responses (Whiteside and Guyton 1952, paper III) are all very much dependent on the initial level of autonomic activity and impinging reflex influences. For example quite different gastrointestinal responses occur upon fastigial stimulation depending on whether intestinal (gastric) inhibitory reflexes are operating or not. When such reflexes are active they are subject to fastigial suppression leading to motility increases (paper II, IV, V). If however no such spinal sympathetot reflex activity is going on the same fastigial stimulation may now depress motility by enhancing the inhibitory adrenergic discharge (Fig. 1). Hence when the cerebellar influence on autonomic function is studied it is of utmost importance to have full control of and insight into the level of activity in both the various efferent autonomic pathways and in the subordi-

nated effector systems. Otherwise the results are likely to be very confusing, apparently lacking all regularity and order.

Parallels to somatomotor functions

Although the present knowledge about cerebellar autonomic control is only fragmentary as outlined above, it seems reasonable to assume that it operates according to similar though not necessarily identical principles as those utilized in the somatomotor coordination. The unique and very uniform neuronal architecture throughout the cerebellum speaks in favour of such a view. However, there are certainly several points where the organization of autonomic and somatomotor efferent control systems differ, even if these two systems no doubt show many close similarities.

To effectuate coordination of somatomotor movements the cerebellum receives information from a variety of somatic sensory receptors and from higher centres as well. Also efferents from visceral organs have been found to project on the cerebellum (Widén 1955; Newman and Paul 1966a, 1969; Langhof, Höppener and Rubia 1973), converging on the same population of cerebellar neurons as the somatic efferents (Bremer and Bonnet 1951; Newman and Paul 1966b). Moreover, the background activity in somatomotor reflex arcs can modify cerebellar somatomotor influences (Maruzzi 1936) as is also the case with background reflex activity in autonomic responses to cerebellar stimulation. Rebound phenomena, well known from experiments on cerebellar somatomotor control, are seen also in association with autonomic influences, e.g. on the bladder (paper III). In addition, one and the same cerebellar stimulation may influence both the somatomotor and autonomic components of combined patterns, like those seen in sham rage (Zanchetti and Zoccolini 1954), defecation (paper III) or the oral behaviour which was predominantly associated with cardiovascular adjustments (paper V). Many situations, like physical activity, emotional behavioural patterns, etc., require concomitant integrations of complex somatomotor and autonomic events which must be well synchronized and timed to occur in proper sequences. Presumably the cerebellum participates in such complex coordinations and the present results lend support to such a view, illustrating some of the principles for the cerebellar modulation combined of autonomic and somatomotor adjustments.

Thus, there is much to indicate that the cerebellum is involved in autonomic regulation and further that such control is carried out according to similar principles as those involved in the somatomotor coordination, though presumably with adaptations according to the somewhat different functional and anatomical characteristics of the two systems.

SUMMARY AND CONCLUSIONS

The present study was carried out to investigate cerebellar control of autonomic functions. For this purpose stimulation of the cerebellar fastigial nucleus seemed to be a suitable approach since the cerebellar influence on bulbo-spinal mechanisms is conveyed via the axonal connections of the nuclear neurones. This cerebellar nucleus receiving its cortical connections predominantly from the medially located vermis region contains in its rostral pole a restricted area which upon stimulation produces pressor responses and from which also a number of other autonomic influences and behavioural changes could be induced as outlined below:

1 Fastigial stimulation was found to suppress the cholinergic vasodilatation in skeletal muscle elicited from the hypothalamic defence area. This suppression was due to a fastigial modulation of activity in the central pathways mediating the cholinergic vasodilatation but whether the effect was exerted at the hypothalamic mesencephalic medullary or spinal relay stations is not clear. The adrenergic links of the defence reaction were on the other hand potentiated by fastigial stimulation. Hence the fastigial impact on the defence reaction was a differentiated one implying both facilitation and inhibition. Some functional implications of this fastigial interaction with the defence reaction were discussed.

2 Intestinal motility was influenced from the fastigial nucleus but differently so in laparotomized cats compared with cats not subjected to any acute abdominal surgery or irritation. Thus in laparotomized cats both excitatory and inhibitory [junal] responses were recorded while the ileum and colon uniformly responded with contraction. Abdominal surgery reflexly induces inhibitory adrenergic discharge to the gastrointestinal tract. The contractions recorded in the ileum and the colon following fastigial stimulation were of all likelihood due to a fastigial suppression of such prevailing inhibitory adrenergic discharge. A similar fastigial suppression of sympathetic gastrointestinal discharge was obtained when intestino-intestinal inhibitory reflexes were induced by intestinal distension or by afferent mesenteric nerve stimulation.

In contrast when intestinal motility was recorded atraumatically fastigial stimulation induced inhibition of ileal motility then reflecting a fastigial facilitation of sympathetic activity to the intestine. If however in these cats a high background sympathetic gastrointestinal discharge was initiated e.g. by abdominal trauma then fastigial stimulation instead suppressed this sympathetic discharge hereby changing the ileal motility response pattern into one of excitation.

3 Fastigial stimulation was potent in influencing both somatomotor and autonomic reflex adjustments involved in defecation. Thus the straining movements as well as the parasympathetically conveyed increases in colonic blood flow and motility were found to be strongly suppressed by concomitant fastigial stimulation. The micturition reflex as well was influenced from the fastigial nucleus but the direction of the responses depended

on the stimulation site in the fastigial nucleus. Further, these responses to fastigial stimulation were also influenced by background bladder tone suppression occurring when the parasympathetic reflex activity was initially high and vice versa. They were predominantly due to a fastigial modulation of parasympathetic discharge to the bladder.

4. Gastric motility was affected from the fastigial nucleus in various ways depending in each case on prevailing activity in the three efferent autonomic links which affect gastric motility. Inhibitory responses could be initiated by a fastigial increase in adrenergic fibre discharge or/and in adrenal catecholamine release. Excitatory responses could be induced by an increase in vagal cholinergic fibre discharge but also by a fastigial inhibition of a prevailing reflex activity in the vagal relaxatory fibres. Further, fastigial stimulation could induce gastric excitatory responses also by suppression of a prevailing sympathetic inhibitory fibre discharge as in the intestine whenever such activity was present as induced by e.g. abdominal surgery or intestinal distension.

5. In conscious cats fastigial stimulation evoked a well coordinated oral behaviour such as licking, grooming, biting and chewing; sometimes even eating. As in anaesthetized cats, this topical fastigial stimulation induced pre- or responses as well but the oral behaviour was not accompanied by any conspicuous adjustments of gastric function in terms of either motility or acid secretion. When the same stimulations were repeated during chloralose anaesthesia in these chronic animals, gastric responses similar to those described above for anaesthetized cats appeared. Histological examination revealed that the responsive cerebellar area, i.e. the rostral fastigial pole was the same for the behavioural changes as for most of the autonomic responses reported in the present investigation.

It is thus possible to induce a variety of autonomic effects from a restricted fastigial area involving virtually all links of the parasympathetic system and the sympathetic system including the adrenals. The results therefore indicate that the cerebellum is involved in the regulation of a variety of autonomic functions perhaps according to principles similar to those valid for cerebellar somatomotor control. The cerebellar influence is not only confined to such selected autonomic responses closely associated with e.g. particular somatomotor adjustments such as the defence reaction, the feeding response, the act of defecation etc. but appears to affect virtually all types of autonomic mechanisms including reflexes engaged in bulbo-spinal homeostatic mechanisms and resulting in cardiovascular, gastrointestinal or urinary bladder adjustments. Probably the cerebellar influence takes the form of a modulating and fine-adjusting influence which is exerted mainly at spinal and bulbar levels but it appears likely that cerebellar autonomic interactions may occur also at higher levels by means of e.g. fastigial projections on hypothalamic and limbic structures.

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Extrinsic Nervous Control of the Ileo-cecal Sphincter in the Cat

BY

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SUPPLEMENTUM 426

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This thesis is mainly based on the following papers which will be referred to in the text by their Roman numerals

- I. Pahlin, P E. and J Kewenter The direct sympathetic nervous control of the cat ileo-cecal sphincter *Amer J Physiol*. Submitted for publication.
- II Pahlin, P E. and J Kewenter Adrenoceptors in the cat ileo-cecal sphincter and intestine studied *in vivo* *Amer J Physiol* Submitted for publication.
- III. Pahlin, P E. and J Kewenter The vagal control of the ileo-cecal sphincter in the cat. *Acta physiol scand*. Submitted for publication.
- IV Pahlin, P E. and J Kewenter Reflexogenic contraction of the ileo-cecal sphincter in the cat following small or large intestinal distension. *Acta physiol. scand* In press.

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INTRODUCTION

Anatomy and function

The gastrointestinal sphincters separate portions of the alimentary canal which differ in anatomy and physiology. An adequate sphincter function can play an important role in preventing pathological conditions, both in the digestive tract and in the body as a whole.

A sphincter is a muscle which surrounds and serves to close an orifice. This definition covers the major functions of the gastrointestinal sphincters i.e. to keep the gastrointestinal contents in one part of the alimentary canal for an appropriate time by preventing too rapid passage or preventing regurgitation. The sphincter of Oddi has a specific function by regulating the bile flow to the intestine but also prevents regurgitation of duodenal contents into the bile ducts.

The gastrointestinal sphincters cannot always be identified anatomically e.g. the gastrooesophageal sphincter in man (Higgs *et al.* 1965). They might, however, be identified by some of their characteristics and different definitions of these characteristics have been proposed. Thus, some physiological characteristics of a gastrointestinal sphincter were defined by e.g. Cohen *et al.* (1968) and Fisher and Cohen (1973): a) A sphincter exhibits an intraluminal pressure greater than that of the cavities separated by the sphincter b) Appropriate stimulation proximal to the sphincter results in a consistent fall in the elevated pressure within the sphincter c) Appropriate stimulation distal to the sphincter results in a prompt rise of the sphincteric pressure. Pharmacologically the gastrointestinal sphincters have been characterized *in vitro* by Bass *et al.* (1970). Sphincteric tissue contracts with adrenaline whereas non sphincteric tissue does not. Thus, although a gastrointestinal sphincter cannot always be identified anatomically there are physiological or pharmacological characteristics by which they can be defined.

The first anatomical description of the ileal termination into the colon was made by Variolus in 1573, but it was Bauhin whose name became associated with this anatomical structure although he did not describe it until 6 years after Variolus. (For historical notes see DiDio and Anderson 1968). However it was Bauhin who, when describing the ileal termination into the colon, was the first to use the term "valvula" which is defined as a membranous fold in a canal or passage which serves to prevent backward reflux of the contents. The concept of an "ileo-cecal valve" dates back to Bauhin's description. It was not until the classical study by Elliott (1904) that a sphincteric function was attributed to the ileo-cecal junction.

Earlier investigations were made in cadavera. When the ileo-cecal junction was studied in living subjects there were no signs of any membranous folds. In the cat the ileal termination in the colon has a similar appearance as in man. Thus, in both the cat and in man the terminal ileum protrudes into the colon and is therefore surrounded by the colonic wall (Elliott 1904 Rosenberg and DiDio 1969) Elliott (1904) investigated the anatomical arrangement of the ileo-cecal junction of the cat and found that the circular muscular coat of the ileum was considerably thickened over about the last 10 mm of its course whereas the longitudinal layer was very thin. Gazet (1968) compared the combined thickness of the circular muscle of the normal ileum and colon with that of the ileo-cecal junctional zone in man, the cat, the dog and the rhesus-monkey. In man, the cat, and the dog there was a considerable thickness of the circular layer in the ileo-cecal junctional zone, but in the rhesus monkey no difference was found.

As mentioned, Elliott (1904) was the first to point out from physiological studies in cats, that the ileo-cecal junction was guarded by a sphincter. In recent years Kelley Jr *et al* (1966) and Cohen *et al* (1968) have confirmed that the ileo-cecal junction in the dog and in man respectively have the physiological characteristics of a gastrointestinal sphincter. This has also been shown pharmacologically *in vitro* in several species e.g. the cat, the guinea-pig, the rabbit, the dog, the rhesus monkey and man (Munro 1951 Reddy 1960 Gazet and Jarrett 1964 Bass *et al* 1970).

According to Alvarez (1948), the ileo-cecal sphincter (ICS) has two purposes: firstly to prevent reflux from the colon into the ileum and secondly to prevent too rapid passage of food residues through the terminal segment of the small bowel.

Two different mechanisms for a reflux preventing function of the ICS have been discussed: a pure valvular mechanism (Hammer 1927 Friedell and Wakefield 1941 Fleischner and Bernstein 1950) and a sphincteric mechanism (Elliott 1904 Katsch 1913 Gazet 1968, Cohen *et al* 1968). Other investigators have considered both mechanisms to be the cause of the competence of the ileo-cecal junction in preventing reflux (Hromada 1921 Ulin *et al* 1956, Lenz 1964).

A reflux of barium into the ileum during the course of a barium enema can be accomplished in about 90% of patients (Fleischner and Bernstein 1950, Miller 1965). Rendleman *et al* (1958) found that there was an increasing incompetence with increasing pressure of the enema. These findings can hardly be explained if there is a valvular effect. Increasing pressures would then by definition close the valve more tightly. A sphincteric mechanism accords better with the above findings. The sphincter yields to the pressure in the colon. During barium enema the pressure is usually high and may exceed 90 cm H₂O. This might explain the high degree of sphincter incompetence found in these studies. Under physiological conditions the pressure rarely exceeds 50 cm H₂O (Kock *et al* 1968).

Whether the ICS also serves to regulate the entrance of the ileal contents into the colon is not quite clear. Kalser *et al* (1960) and Stahlgren *et al* (1962) considered the ICS to be of great importance for the normal small intestinal transit time.

Singleton *et al.* (1964) however were able to register only a slight decrease of the transit time by excision of the ICS or 50% of the small intestine, but when both parts were excised there was a marked decrease in the average transit time. Similarly Didio and Andersson (1968) considered the combination of the motility in the distal ileum and the resistance to flow offered by the ICS to be important for the regulation of the passage of the gastrointestinal contents from the ileum into the caecum.

The sympathetic and parasympathetic innervation of the ICS

Elliott (1904) found that the removal of the spinal cord permanently abolished the ability of the sphincter to keep the contents of the ileum and the colon apart, indicating that the sphincteric muscles are dependent upon an intact extrinsic nervous system for adequate function. However, Tönnis (1924) considered that the exogenous nerves to the ICS exerted a regulatory control on an autonomically functioning sphincter but that the sphincter could function although these nerves had been cut.

From previous studies on the extrinsic nervous regulation of the ICS it is known that stimulation of the splanchnic nerves elicits an increase of pressure within the ileo-caecal junction in cats and dogs due to contraction of the ICS (Elliott 1904 Dale 1906, Hinrichsen and Ivy 1931 Jarrett and Gazet 1966). The lumbar colonic nerves which are mainly postganglionic fibres of the lumbar sympathetic outflow have not been shown to take part in the innervation of the ICS. Elliott (1904) found no changes in the tone of the ICS upon stimulation of these nerves.

The influence of the vagal nerves upon the ICS has been sparsely investigated. Elliott (1904) discussed the possibility of the contraction of the ICS following cervical vagal stimulation being due to anaemia in the intestine caused by the concomitant inhibition of the heart. Hinrichsen and Ivy (1931) recorded in dogs a biphasic response following electrical stimulation of these nerves; relaxation followed by contraction. In cats, Jarrett and Gazet (1966) noted different responses depending upon the frequency of the stimulation, contraction was elicited at high frequencies (50 imp/s) and relaxation at low frequencies (5 imp/s). In dogs, however only contractions were recorded. As the vagal nerves convey excitatory fibres to the proximal colon (Stavney *et al.* 1963 Hultén 1969) it cannot be excluded that the vagally induced contraction of the ICS might be partly or entirely due to a secondary squeezing effect of the colonic wall surrounding the sphincter. Thus, the influence of the vagal nerves upon the ileo-caecal sphincter muscle must be considered unclear.

The autonomic transmitter mechanism

The autonomic transmitter mechanism involved in the responses of the ICS to stimulation of the sympathetic and parasympathetic nerves has been little studied. The classical concept of the sympathetic system being strictly adrenergic and the

parasympathetic one strictly cholinergic has been shown to have many exceptions (see Christensen 1968). Thus the sympathetic nerves may contain cholinergic fibres (Thomas and Baldwin 1968, Hultén 1969 Persson 1973) and the parasympathetic nerves adrenergic fibres (Martin *et al* 1974). Furthermore, it has been clearly demonstrated that motor responses obtained in the gastrointestinal tract following autonomic nerve stimulation can be elicited by a non-cholinergic, non-adrenergic mechanism (for review see Burnstock 1972).

The work of Dale (1906) indicated an adrenergic mechanism as the cause of contraction of the ICS following splanchnic nerve stimulation in the cat. Studies *in vitro* on muscle strips from the ICS in different species (rabbit, guinea pig, cat, dog and man) have shown that these strips contract with both adrenergic and cholinergic drugs (Reddy 1960 Reynolds *et al* 1967 Gazet and Jarrett 1964 Bass *et al* 1970). This indicates adrenergic as well as cholinergic excitatory receptors within the ICS. It has been shown, also *in vitro* that the excitatory response to the adrenergic drugs was mediated by α -receptors (Reddy 1960, Gazet and Jarrett 1964 Reynolds *et al* 1967). Inhibitory adrenergic β -receptors in the ICS were postulated from *in-vitro* studies in cats, dogs, man and rhesus-monkeys by Gazet and Jarrett (1964) and in guinea-pigs by Reynolds *et al* (1967). Reddy (1960) found that isoprenaline in small doses relaxed muscle strips from the rabbit ICS while large doses elicited a contraction. Both effects were blocked by β receptor blockade, indicating that both relaxation and contraction could be mediated via the β -receptors. In the small and large intestines the α -receptors are considered generally to be inhibitory (Ahkquist and Levy 1959 Furchgott 1960 Brody and Diamond 1967). A difference in function *in vitro* of the α -receptors in the ICS and the intestinal smooth muscles is thus indicated. Therefore a further study *in vivo* of the functional significance of these *in-vitro* results seemed to be justified.

Reflex activation of the ICS

Reflex activation of the ICS by distension of different parts of the intestinal tract has been reported. Hinrichsen and Ivy (1931) noted contractions upon distension of the stomach and the small and large intestines in dogs while Chang and Hsu (1942) upon distension of small intestinal loops in dogs, recorded both contractions and relaxations in the sphincteric area depending upon the tone of the sphincter at the time of the distension. The former authors considered that the reflex was dependent upon both the splanchnic and vagal nerves being intact while the latter authors considered the splanchnic nerves to be the main pathway for the reflex.

AIMS OF THE INVESTIGATION

From previous studies it may be concluded that the junctional zone between the ileum and the colon is guarded by a sphincter. Only a few studies have dealt with the extrinsic nervous control of the sphincter and the results are sometimes conflicting. Furthermore, few data concerning the autonomic mechanisms involved in the nervous control of the ICS are available.

The aims of the present investigation were therefore to study

1. The direct splanchnic and lumbar colonic nervous control of the ICS.
2. The adrenergic α - and β receptor mechanisms in the ICS.
3. The direct vagal control of the ICS.
4. The nervous pathways and peripheral autonomic transmitter mechanism involved in the reflex activation of the ICS following intestinal distension.

MATERIAL AND METHODS

Experiments were performed on 181 adult cats of both sexes. The number of animals used in the various series is given in each paper. Some cats were used for experiments in several different series. The cats were fasted for about 24 hours before the experiments, but were allowed free access to water. In all animals anesthesia was induced with ether and maintained by an intravenous injection of chloralose (50 mg/kg b.w.). A tracheal cannula was inserted to maintain free airways and all animals breathed spontaneously except in a few experiments (IV) in which a respirator was used. The blood pressure was continuously recorded by means of a mercury manometer connected to a femoral artery. Great care was taken to keep the animal at normal body temperature.

Operative procedure. Laparotomy was performed through midline incision. In most cats the small intestine, apart from the parts used for distension (IV) and for recording intestinal motility was extirpated. The colon was left in place. As catecholamine secretion from the adrenal medulla is known to be a potent intestinal inhibitor (Kock 1959) the vessels of these glands were ligated in the beginning of all experiments except for two (IV) in which ligatures were performed during the course of the experiments.

Recording of transsphincteric flow (I—IV). Fig. 1 shows a schematic drawing of the method for recording transsphincteric flow. The ileum was divided about one cm proximal to the sphincter and a "crushing" ligature was tied around the colon one to two cm distal to the sphincter. In this way the ileo-cecal region was isolated from the adjacent gut but with the mesenteric vascular and nerve supply intact. A tube was introduced into the remaining ileum distal to the transection and fixed with a ligature at the oral end of the sphincter. Normal saline, thermostatically regulated to be at body temperature, was led from a reservoir to the sphincter via the tube. The saline passing the sphincter was drained from the cecum by means of a funnel introduced with its wider end through an incision in the antimesenteric border of the gut opposite to the sphincter. The narrow end of the funnel was connected to a Gaddum recorder (Gaddum 1929). Volume changes in the recorder reflected changes in the flow through the sphincter. The volumes were registered via a piston-recorder on a smoked drum. From the Gaddum recorder the saline was

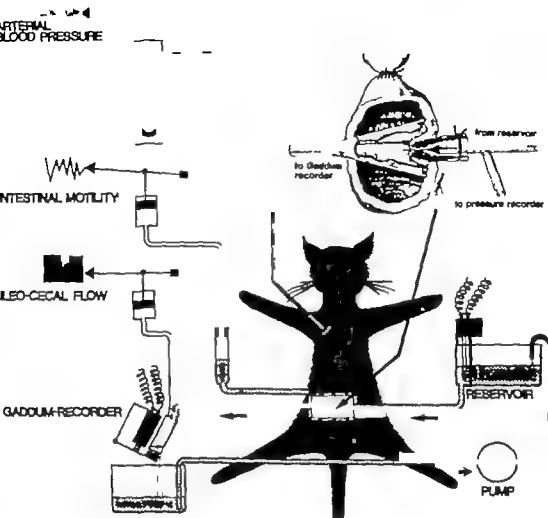


Fig. 1 Schematic drawing of the experimental set up. For explanation see text. (Pressure recorder not illustrated in the figure).

continuously recirculated into the reservoir. As the reservoir had a large cross-sectional area the inflow pressure, i.e. the height of the saline level over the sphincter could be kept almost constant. The inflow pressure was usually kept around 5 cm H_2O at which pressure the flow capacity of the inflow tube was 3—4 times greater than the maximal flow recorded through the sphincter. The reservoir could be adjusted to different heights so the sphincter could be perfused at different pressures.

Recording of opening pressure (I and III) The opening pressure was defined as the pressure needed to induce a flow through a closed sphincter. This pressure was measured from a small side-branch to the perfusion tube just proximal to the sphincter (Fig. 1) and was registered via a pressure receptor on a kymograph (Mingograph, Siemens-Elema, Stockholm). When the sphincter was closed, the perfusion pressure was raised by elevating the reservoir until the flow just started. The pressure recorded at that moment was thus called the opening pressure.

Recording of intestinal motility (I—IV) The small and large intestinal motility was recorded with a volume recording technique previously described (e.g. Kock 1959). About 10 cm long intestinal loops from the proximal jejunum, the ileum just proximal to the ICS and the colon just distal to the ICS were studied. The loops were isolated from the adjacent gut but with their mesenteric nerve and blood supply intact.

Nerve stimulation (I—IV) The following nerve trunks were divided and stimulated electrically in the efferent direction, the two major splanchnic nerves subdiaphragmatically (I—IV) the nerves along the superior mesenteric artery here called "the periaarterial nerves" (I and II) the nerves along the inferior mesenteric artery known as "the lumbar colonic nerves" (e.g. Garry 1933) (I and II) and the cervical vagal nerves (III).

Square wave pulses were delivered from a Grass stimulator (S4E). Supramaximal currents were used (4—12 V 3—5 ms) 4—8 imp/s was usually used but the frequency was varied over a wide range (2—50 imp/s) in some experiments. Stimulation trains between 30—60 seconds were usually used.

Intestinal distension (IV) Intestinal distension was elicited from a jejunal, ileal or colonic loop of about 10—15 cm in length. The loops were isolated from the adjacent gut but with the mesenteric nerve and vascular supply intact. The loops were distended with body-warm saline from a reservoir that could be adjusted to different heights. Distensions of 30—60 seconds duration and at intervals of 10 minutes were usually applied. The pressures used were 30—100 cm H₂O.

Nerve sections (IV). To elucidate the possible nervous pathways for the reflex activation of the ICS upon intestinal distension the following nerves were cut: the vagal nerves at cervical level the pelvic nerves at the outlet from sacrum the two major splanchnic nerves subdiaphragmatically the lumbar colonic nerves along the inferior mesenteric artery.

Spinal anesthesia (IV) In order to achieve a preganglionic sympathetic blockade, spinal anesthesia was performed. The anesthetics were injected via a thin polyethylene catheter introduced intradurally in the caudal direction through a cervical laminectomy.

Drugs. (I—IV) Drugs were administered to produce blockade as follows. muscarinic cholinergic receptors. atropine (Atropine sulphate, Merck) 0.1—1 mg/kg b.w. i.v. (I, II, III and IV).

Ganglionic transmission. chlorisondamine (Ecolid® Ciba) 2 mg/kg b.w. i.v. (I)

Adrenergic transmission. guanethidine (Ismelin® Ciba) 1—5 mg/kg b.w. i.v. (I, III and IV)

Adrenergic α -receptors: phenoxybenzamine (Dibenyline® Smith, Klein and French) 1—15 mg/kg b.w. i.v. (II and IV).

Adrenergic β -receptors: propranolol (Inderal® ICT) 1—2 mg/kg b.w. i.v. (II and IV).

The following sympathomimetic drugs were used adrenaline 0.1—2 μ /kg b.w., noradrenaline 0.1—7 μ /kg b.w., phenylephrine 0.3—11 μ /kg b.w., and isoprenaline 2—100 μ /kg b.w. These drugs were administered either intravenously through a femoral vein or intraarterially in the retrograde direction through a small branch to the superior mesenteric artery (II and III).

Spinal anaesthesia (IV) was induced with Tetracaine grave® (Apoteksbolaget, Sweden) 10 mg/ml or mepivacaine chloride (Carbocain® Nobel Pharma) 20 mg/ml. Both drugs were administered in an amount 1—2 ml intradurally.

METHODOLOGICAL CONSIDERATIONS

In previous studies in which the extrinsic nervous control of the ICS has been investigated pressure registration techniques have usually been used (Elliott 1904 Hinrichsen and Ivy 1931 Jarrett and Gazet 1966). However when pressure receptors are used it may be difficult to keep the receptor within the sphincteric region (Elliott 1904). Furthermore, the ICS has a flow regulating function, and it was therefore thought of interest to use a technique by which the flow through the sphincter could be studied. The method described in the present report was suitable for this purpose. (1) Changes in the transsphincteric flow could be recorded continuously throughout the whole experiment. The changes of the flow at a constant perfusion pressure were due to changes in the tone of the ileo-cecal junction as the inflow tube could easily be placed at the proximal end of the sphincter. Furthermore, the perfusion tube and the funnel used to drain the ICS had a flow capacity which was 3—4 times larger than the maximal flow recorded through the ICS at an inflow pressure of about 5 cm H_2O .

The perfusion pressure could be kept constant as the cross-sectional area of the saline reservoir was large and as the saline was continuously recirculated. The pressure was usually set to about 5 cm H_2O . At this pressure the flow through the sphincter was easily maintained. The normal resting pressure within the cat ileo-cecal junction is not known. In unanesthetized dogs (Kelley Jr *et al.* 1965) and in man (Cohen *et al.* 1968) the average resting pressure was as high as 19 cm H_2O and 20 mm Hg respectively above the ambient intestinal pressure. Provided that there are only minor species differences in the resting pressure, the tone within the ICS in the present experiments was comparably low. The cause of this low tone is unclear. However anesthesia has been shown to reduce the pressure greatly within the lower oesophageal sphincter (Code and Schlegel 1968) and in the pharyngo-oesophageal sphincter (Levitt *et al.* 1965). This might also be the cause of the low tone of the ICS in the present studies. As the flow pattern was the same whether the extrinsic nerves were intact or not, influence from these nerves does not seem to be the cause of the low sphincteric tone.

The temperature of the perfusion saline was of great importance. If the temperature was set at about $+20^{\circ}C$ a high perfusion pressure (sometimes about 50 cm H_2O) had to be used to maintain a transsphincteric flow while with the saline at body temperature the flow could easily be maintained at a low perfusion pressure (about 5 cm H_2O).

As previously stated, the terminal ileum protrudes into the colon and is thus surrounded by the colonic wall (Elliott 1904). Changes in the transsphincteric flow might therefore depend upon changes either in the tone of the sphincter itself, the tone of the colon, or both. However, the motor activity of the intestines just proximal and distal to the sphincter was recorded simultaneously with the transsphincteric flow in some of the experiments in order to compare the motor activity in the intestine and the ICS.

RESULTS AND COMMENTS

Sympathetic nerve stimulation (I—IV)

Efferent electric stimulation at physiological frequencies (2—12 imp/s) and supra-maximal currents of the distal ends of the divided major splanchnic nerves, the periaarterial nerves or the lumbar colonic nerves elicited reduction or inhibition of the transsphincteric flow concomitant with an inhibition of the ileal and proximal colonic motility. As the flow reduction was noted within a few seconds and any influence from the suprarenal glands was excluded, it was concluded that the effect of sympathetic nerve stimulation was due to an excitatory nervous effect on the ICS. In some of the experiments a slight increase of the transsphincteric flow could be recorded during the first few seconds of stimulation. This flow increase could not be regularly elicited although the stimulation parameters were varied over a wide range. The results indicate that the main influence of the sympathetic nerves on the ICS is excitatory which is in accordance with most previous studies (Elliot 1904, Dale 1906, Hinrichsen and Ivy 1931, Jarrett and Gazet 1966). Excitatory fibres within the lumbar colonic nerves to the ICS seem not to have been previously reported.

Autonomic blockade (I—II). The splanchnic, periaarterial and lumbar colonic nerves were also stimulated after intravenous administration of atropine (0.2 and 1 mg/kg b.w.) to investigate whether ICS contraction was due to an activation of cholinergic fibres within these nerves (I). The lower dose of atropine did not block the excitatory effect of the ICS to the nerve stimulation, while the larger dose caused a transient blockade of the response. A similar transient blockade by atropine (1 mg/kg b.w.) of the excitatory effect of noradrenaline on the ICS was also found in 6 cats investigated (Fig. 2). After 15—60 minutes, splanchnic nerve stimulation, as well as noradrenaline infusion elicited the same response on the ICS as before atropine had been given. A similar antagonistic effect of atropine on the excitatory response in the sphincter of Oddi to sympathetic nerve stimulation *in vivo* in the cat has been reported by Persson (1973) and in the lower oesophageal sphincter by Clark and Vane (1961). Furthermore, Christensen and Daniel (1968) found that atropine blocked the excitatory response to noradrenaline in an *in-vitro* preparation from the smooth muscle of the lower oesophageal sphincter.

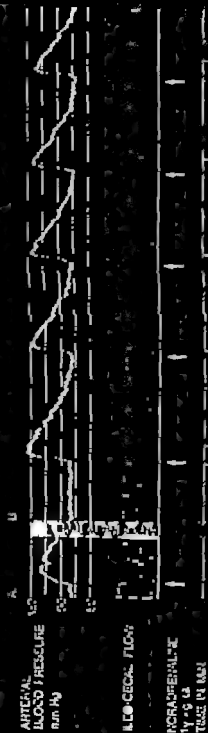


Fig. 2. Effect of intra-arterial injection of noradrenaline on the transphincteric flow before and after atropine. Note that a transient blockade of the response was obtained.

This blocking action of atropine is different from the muscarinic effect of the drug causing the blockade of the excitatory response in the stomach (Martinson 1965) the small and large intestines (Kewenter 1965 Hultén 1969) and the ICS (II) by vagal nerve stimulation. In these experiments, even small doses (0.1—0.5 mg/kg b.w.) of atropine blocked the effect of vagal stimulation for several hours. It does not seem likely therefore, that the short lasting suppressant effect of large doses of atropine on the excitatory response of the ICS following sympathetic nerve stimulation is due to an anticholinergic mechanism. Non specific effects of atropine in the higher dose (10 mg/kg b.w. i.v.) might be caused by an antiadrenergic effect of the d isomer of hyoscyamine in atropine as reported by Luduena and Brann (1966). Furthermore, atropine in large doses is also known to have a slight ganglion-blocking action (Marrazzi 1939). As the response in the ICS to periaarterial and lumbar colonic nerve stimulation, which nerves are considered to be mainly post ganglionic, was reduced in the same way as the effect of splanchnic nerve stimulation this latter explanation seems not to be relevant.

The effect of autonomic blocking drugs on the initial slight increase of the transsplanchnic flow at splanchnic stimulation could not be evaluated due to the irregularity in this response. As the responses were also seen after atropine, however the flow increase seems not to be dependent on a cholinergic mechanism.

Guanethidine completely blocked the excitatory response of the ICS to sympathetic nerve stimulation, which indicates an adrenergic transmitter mechanism (I). This was further supported by the results reported in paper II, in which it was shown that adrenaline, noradrenaline and phenylephrine also elicited a contraction of the ICS. The sphincteric excitatory responses obtained by both the sympathomimetic amines and sympathetic nerve stimulation were blocked by the α -receptor blocking agent phenoxybenzamine, while they remained after β receptor blockade.

After propranolol the excitatory response in the ICS following injection of sympathomimetic amines (adrenaline, noradrenaline, phenylephrine) was enhanced in more than 2/3 of the experiments (II). Following adrenergic nerve stimulation a corresponding increase of the sphincter contraction after propranolol was seen in about 1/3 of the experiments (II). The increased response might be due to an unmasking of the excitatory α -receptor response through blockade of inhibitory β receptors by propranolol. This effect of β blockers has been proposed by Ahlquist (1968). Inhibitory β receptors could be further established in a series of experiments showing that isoprenaline relaxed the ICS during a sphincter contraction elicited by adrenergic nerve stimulation (II). This increased transsplanchnic flow following isoprenaline was not due to relaxation of the intestine surrounding the sphincter as the intestine was already relaxed by the adrenergic nerve stimulation. The inhibitory effect of isoprenaline was blocked by propranolol. These studies strongly suggest the presence of inhibitory β -receptors in the ICS.

The inhibition of intestinal activity elicited by adrenergic stimulation is mediated via both α - and β receptors (Ahlquist and Levy 1959 Furchgott 1960 Bucknell

and Whitney 1964 Brody and Diamond 1967). In paper II it was shown that neither phenoxybenzamine nor propranolol blocked the inhibitory motor response of the small and large intestinal loops following adrenergic nerve stimulation. Both an α - and β -receptor blocker had to be administered to obtain complete blockade of the inhibitory motor response elicited by nerve stimulation. This confirms the concept of both α - and β receptors as mediators of adrenergic inhibition of the intestine. A functional difference between the α -receptors in the ICS and the intestine is thus indicated.

From *in-vitro* studies on the guinea pig ileum Kosterlitz and Watt (1965) suggested that the inhibitory α -receptors are located within the intramural cholinergic ganglion cells while the inhibitory β receptors are situated on the muscle cell. If the effect of the adrenergic nerves on intestinal motor activity is confined exclusively or almost exclusively to the cholinergic ganglion cells, as suggested by histochemical and physiological studies (Norberg 1964 Jacobowitz 1965 Kewenter 1965 Jansson and Martinson 1966, Hultén 1969), one would then expect that the inhibitory response of the intestine, following sympathetic nerve stimulation, would be blocked by phenoxybenzamine only provided that the suggestion by Kosterlitz and Watt (1965) is true. The fact that such a blockade was not obtained might be explained by e.g. direct adrenergic fibres to the intestinal smooth muscle eliciting inhibition *via* unblocked β -receptors. Adrenergic fibres within the circular smooth muscles of the intestine have been demonstrated histochemically in recent years by e.g. Costa and Gabella (1971) and Silva *et al* (1971). Another possibility might be an overflow of the transmitter from adrenergic nerve endings to the β receptors at the muscle cells (Paton and Vizi 1969). A third possibility is that the α -receptors within the intestine have not been completely blocked although a total blockade of the effect on the blood pressure and sphincter following phenoxybenzamine administration was indicated. Irrespective of the mode of action it may be concluded that the α -receptors within the ICS and the intestine produce a different response, contraction and inhibition respectively when they are activated by means of splanchnic or lumbar colonic nerve stimulation. Furthermore, the present results indicate that the inhibitory adrenergic influence on the motor activity of the small intestine is not totally mediated *via* α -adrenergic receptors on the intramural cholinergic ganglion cells.

In general, a sympathetically innervated organ has a preponderance of receptors of one type, although a small proportion of the other type may also be present (Innes and Nickerson 1968). In this study contraction of the ICS was the dominating response to sympathetic nerve stimulation as well as to catecholamine infusion which responses were blocked by phenoxybenzamine. This indicates that excitatory α -receptors are in preponderance within the ICS.

To summarize the experiments in I and II have shown that sympathetic nerve stimulation as well as adrenaline, noradrenaline and phenylephrine elicits a contraction of the ICS. As this response was blocked by guanethidine and phenoxy-

benzamine but not by propranolol an adrenergic α -receptor mechanism as cause of the response is strongly indicated. The presence of inhibitory β -receptors within the ICS is also suggested. Both phenoxybenzamine and propranolol had to be administered to block the adrenergic inhibitory effect on the intestinal motor activity

Vagal nerve stimulation (III)

Efferent cervical vagal nerve stimulation with physiological frequencies and supra maximal currents was shown to reduce or abolish the transsphincteric flow. In contrast to the effect of sympathetic stimulation this flow decrease was usually concomitant with an increase in motor activity in the small intestine and more seldom in the proximal part of the large bowel. The reduced transsphincteric flow might thus be due either to a contraction of that part of the intestine that surrounds the sphincter or to a contraction of the sphincter itself or both. To elucidate whether the decreased transsphincteric flow was due to sphincteric contraction or not, the vagal stimulation was repeated during continuous sympathetic nerve stimulation, which is known to suppress the excitatory effect of vagal stimulation on the intestine (Kewenter 1965, Hultén 1969). It was then found that vagal stimulation stopped the transsphincteric flow although no concomitant ileal contraction could be recorded. There were thus indications that the flow stopped because of a contraction of the sphincteric muscle.

The hypothesis of an excitatory vagal nervous effect on the sphincteric muscle was further supported by the results obtained with vagal stimulation after administration of guanethidine. Before this drug an increased motor activity in the colon was only obtained in a few experiments following vagal stimulation, while there was a stop of the transsphincteric flow in the majority of the experiments. In contrast, after guanethidine vagal stimulation elicited a pronounced contraction of the colon in the majority of the experiments but no or a delayed, reduction of the transsphincteric flow. It therefore seems unlikely that the colonic motor activity interferes with the transsphincteric flow. It can of course be argued that the colonic motor activity was recorded distal to the sphincter region but there are no indications of a qualitative difference in the influence of the vagal nerves on the caecum and the proximal part of the colon.

In order to reveal any inhibitory fibres within the vagal nerves to the ICS stimulation was repeated during continuous intra arterial infusion of noradrenaline in order to create an increased sphincter tone on which possible inhibitory fibres could act. The opening pressure was repeatedly recorded in this series of experiments. As shown in Fig. 3 the opening pressure gradually decreased despite continuous noradrenaline infusion. When the vagal nerves were stimulated the opening pressure increased once again. A decrease of opening pressure upon vagal stimulation was never obtained, indicating that no relaxatory fibres are present in the vagus to the ICS.



Fig. 3. Effect of vagal nerve stimulation on the opening pressure during intra-arterial noradrenaline infusion. Note the gradually decreasing opening pressure despite the continuous infusion. Vagal stimulation again elicited an increased opening pressure.

Catecholamines have been shown to suppress the output of acetylcholine in *in-vitro* preparations from the colon and ileum subjected to nerve stimulation (Beal *et al* 1969 Paton and Vizi 1969) and to suppress the intestinal excitatory motor response following vagal stimulation *in vivo* (Kewenter 1965). The increase in opening pressure upon vagal stimulation during noradrenaline infusion therefore supports the assumption that the vagal nerves exert their effect direct on the ICS and not *via* the colonic wall surrounding the sphincter.

Increased pressure in the ileo-cecal junction of the dog following vagal nerve stimulation was also obtained by Hinrichsen and Ivy (1931). In their experiments the contraction was usually preceded by a slight inhibition of the sphincteric tone, an effect that was never observed in the present experiments. In the cat, Jarrett and Gazet (1966) could elicit different responses in the ICS by cervical vagal stimulation. They obtained relaxation at low frequencies (5 imp/s) and contraction at high frequencies (50 imp/s). In the present study only excitatory responses were obtained at frequencies between 2 and 50 imp/s.

Autonomic blockade. Atropine and guanethidine are widely used to block cholinergic and adrenergic nervous transmission respectively. In paper III it was found that atropine blocked all the responses to vagal stimulation but also that guanethidine in most experiments blocked or markedly delayed the contractile response of the ICS to vagal stimulation, while the excitatory effect on the intestinal loops was greatly enhanced.

The results concerning the blockade by both drugs of the vagally induced sphincteric response are difficult to explain. Guanethidine has been shown to have an anticholinergic action in certain nerve-muscle preparations (Boyd *et al* 1963) while atropine in low doses is considered to be a specific anticholinergic agent (Innes and Nickerson 1968). Therefore a cholinergic mechanism seems to be the most plausible cause of the contraction of the ICS upon vagal stimulation.

According to Burnstock (1972) non-adrenergic inhibitory as well as non-adrenergic, non-cholinergic excitatory neurons are present in the small intestine of mammals. In the present study neither excitation nor inhibition of the ICS was obtained after atropine and/or guanethidine upon vagal stimulation. Therefore no indications of the presence of such neurons in connection with the vagal nerves have been obtained.

From the results in III it can be summarized that vagal nerve stimulation causes a decrease of the transsphincteric flow depending, at least partly upon a direct excitatory nervous effect on the ICS muscle. This effect was suggested to be cholinergic.

Termination of adrenergic fibres in the ICS

Excitatory responses in the ICS obtained by both cholinergic and adrenergic nerve stimulation give rise to several alternatives in the mutual relationship between the adrenergic and cholinergic nerve terminals within the intestinal wall. This mutual relationship has previously been discussed (e.g. Christensen 1968, Christensen 1971, Youmans 1972, Burnstock and Costa 1973). At least three alternatives might be considered as possible explanations to the present results (Fig. 4).

In paper I it was shown that the excitatory effect on the ICS following splanchnic stimulation was blocked by the ganglionic blocking agent chlorisondamine (2 mg/kg b.w.) while the contraction could be elicited again when the periarterial nerves, which are considered to be constituted mainly by postganglionic neurons of the splanchnic nerves, were stimulated (Fig. 5 panel A, B and C). This might imply a direct effect of the adrenergic nerves via excitatory α -receptors on the sphincteric muscles (B in Fig. 4) as it was shown in I and II that the contraction of the ICS following periarterial nerve stimulation was blocked by guanethidine (Fig. 5 panel E) and phenoxybenzamine but not by propranolol. There is anatomical support for this assumption as Costa and Gabella (1971) and Silva *et al* (1971) have shown that adrenergic nerves not only terminate in the intramural plexuses as suggested by Norberg (1964) and Jacobowitz (1965) but are also present within the circular muscle layer of all regions of the subdiaphragmatic digestive tract.

However as previously mentioned, Kosterlitz and Watt (1965) suggested that the α -receptors within the intestine are located at the intramural cholinergic ganglion cells. This alternative explanation would imply that postganglionic adrenergic fibres act on the intramural cholinergic ganglion cells within the ICS, as shown in A in Fig. 4. The effect of adrenergic nerve stimulation would then be an increased liberation of acetylcholine at the postganglionic nerve endings as it was indicated in III that cholinergic nerve stimulation elicited a contraction of the ICS. However one must then postulate that such adrenergic synapses would be resistant to chlorisondamine (Fig. 5 panel C).

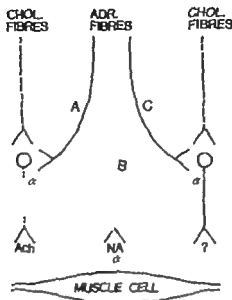


Fig. 4 Three different alternatives for the adrenergic innervation of the smooth muscle cells in the ICS. For discussion see text.

A. Nervous termination on intramural cholinergic ganglia.

B. Nervous termination on splanchnic smooth muscle cells.

C. Nervous termination on intramural ganglion cells with unknown peripheral transmitter

A final common pathway for the cholinergic and adrenergic nervous systems was discussed by Christensen and Daniel (1968). They found that cholinergic as well as adrenergic drugs elicited a contraction of the lower oesophageal sphincter of the cat *in vitro* and that the response to both kind of drugs was blocked by atropine. The excitatory motor response in the ICS following adrenergic nerve stimulation was not blocked by a low dose of atropine and only transiently by a larger dose (Fig. 5 panel D), so that this explanation is unlikely.

A third possible explanation for the adrenergic excitatory motor response in the ICS might be that the adrenergic nerves exert influence *via* α -receptors on non-cholinergic, non-adrenergic nerve fibres within the ICS (C in Fig. 4). Although such fibres are considered generally to be inhibitory (Burnstock 1972), excitatory fibres of this kind have been shown to supply the small intestine of the cat (Day and Warren 1968). Although such an explanation for the excitatory response in the sphincter following adrenergic nerve stimulation cannot be excluded from the present experiments, far too little is known at present about such non-cholinergic, non-adrenergic excitatory fibres to postulate this mechanism as the cause of the contraction of the sphincter.

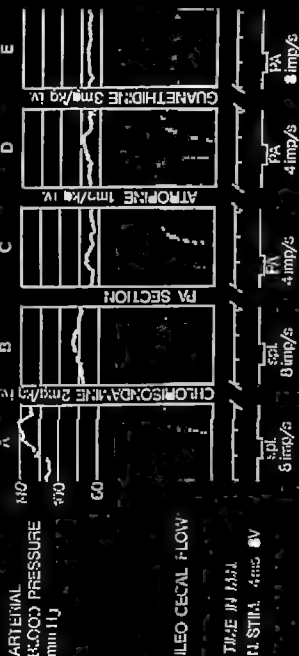


Fig. 5 Effect of splanchnic nerve stimulation before and after chlorisondamine (Panel A and B). Effect of periaarterial nerve stimulation after chlorisondamine, atropine and guanethidine respectively (Panel C, D and E). For details, see text.

Thus, in the light of present knowledge, the suggestion of a direct effect of the adrenergic nerves *via* excitatory α -receptors on the sphincteric muscle seems most reasonable. Such an assumption might imply at least a quantitative difference in the peripheral innervation between the ICS and other gastrointestinal smooth muscles where the adrenergic nerves are considered to exert their effect mainly by an inhibitory action on the intramural pre- and postganglionic cholinergic transmission (Kewenter 1965 Jansson and Martinson 1966, Hultén 1969 Paton and Vizi 1969 Beani *et al* 1969). Such quantitative difference in the location of the adrenergic terminals in the intestine and the ICS might possibly have a relation to the difference in function between the α -receptors in these two parts of the gastrointestinal tract. An alternative explanation is a difference in responsiveness to the transmitter in the smooth muscle cells from different parts of the intestine (e.g. Gazet and Jarrett 1964).

Reflex activation of the ICS by intestinal distension (IV)

Distension of a small or large intestinal loop isolated from the adjacent gut but with the mesenteric vascular and nervous supply intact, decreased or abolished the transsphincteric flow. As there was a concomitant inhibition of intestinal motor activity it was concluded that the reduced transsphincteric flow was due to pure sphincter contraction.

Contraction of the ICS following large intestinal distension is in agreement with the physiological characteristics of a gastrointestinal sphincter as defined by e.g. Cohen *et al* (1968) and Fisher and Cohen (1973) while the contraction of the sphincter elicited by distension of the small intestine is contradictory to their definition in which the tone of the sphincter is considered to be decreased by pre-sphincteric stimulation. Such relaxant responses have been recorded in the ICS of the dog and man following ileal distension (Kelley Jr *et al* 1966, Cohen *et al* 1968). Excitatory responses in the ICS following small intestinal distension, as in the present study were found by Hinrichsen and Ivy (1931) in dogs while Chang and Hsu (1942), also in dogs, obtained contraction when the tone of the sphincter was low and relaxation when the tone was high. In the present study no relaxation of the sphincter could be recorded even when the tone of the sphincter had been raised by either adrenergic nerve stimulation or distension of another intestinal loop.

Nerve section and spinal anesthesia. As the vessels of the suprarenal glands were ligated and the continuity between the intestinal loops and the ileo-cecal region was broken an extrinsic nervous pathway for the reflex was concluded. The pathway for the excitatory intestino-ileo-cecal sphincteric reflex elicited by small intestinal distension was further studied by nerve section and spinal anesthesia. The cranial and sacral parasympathetic outflow could be excluded as mediators of the reflex as this was not affected by section of the vagal and/or pelvic nerves.

Section of the major splanchnic and lumbar colonic nerves, but not one or the other completely or almost completely abolished the response and these nerves were therefore considered as the major pathways for the reflex. This is not in agreement with the findings of Hinrichsen and Ivy (1931) and Chang and Hsu (1942). The former authors considered the reflex to be dependent upon both the vagal and splanchnic nerves, while the latter authors concluded that the splanchnic nerves were the only pathways for the reflex.

Spinal anesthesia totally abolished the reflex. Small intestinal distension is known to elicit inhibition in other parts of the gastrointestinal canal (Johansson and Langston 1964, Jansson and Martinson 1966, Hultén 1969). Johansson and Langston (1964) could clearly show that the intestino-intestinal inhibitory reflex was spinal and not mediated via a synaptic mechanism in the mesenteric ganglia (Kuntz and van Buskirk 1941, Kuntz and Saccomanno 1944). It could further be shown that the reflex was under supraspinal control (Johansson *et al* 1965 and 1968). A similar spinal pathway for the intestino-ileo-cecal sphincter reflex is also indicated from the present study but whether it is under supraspinal control or not was not investigated.

Autonomic blockade As the reflex contraction of the sphincter was not blocked by atropine or propranolol but was blocked by guanethidine and phenoxybenzamine it was concluded that the reflex was mediated via an excitatory adrenergic α -receptor mechanism just as the response following sympathetic nerve stimulation.

Thus, intestinal distension elicits a contraction of the ICS. This excitatory intestino-ileo-cecal sphincteric reflex is proposed to be a sympathetic spinal reflex mediated via an α -adrenergic mechanism.

FUNCTIONAL CONSIDERATIONS

The ICS might have two functions, to prevent regurgitation of material from the cecum into the small bowel and to delay the passage of the ileal contents into the cecum (Alvarez 1948). In the present study almost exclusively contractory effects on the sphincter were obtained both upon nerve stimulation and intestinal distension thus supporting the function of the ICS as being to keep the ileal and colonic contents apart.

However the opening mechanism of the sphincter is not clear. Several factors may contribute to the relaxation of the sphincter e.g. decreased tonic activity in the adrenergic and cholinergic nerves to the sphincter. Activation of possible inhibitory β -receptors in the ICS and/or reflex relaxation of the sphincter similar to that described by Kelley Jr *et al.* (1966) and Cohen *et al.* (1968) are other alternatives as it cannot be excluded that the continuity of the intestine might be of importance in mediating a reflex inhibition of the ICS. An additional explanation for decreased sphincter tone might be the influence of gastrointestinal hormones which may take part in the regulation of intestinal tone and motility (Maikhlouf 1974). Recently gastrin, both endogenous and exogenous, has been shown to decrease the tone of the ICS in man (Castell *et al.* 1970). The effect of the pelvic nerves on the ICS is not known. Inhibitory fibres within these nerves to the sphincter cannot be excluded.

SUMMARY

The influence on the ileo-cecal sphincter (ICS) of electrical efferent stimulation of the splanchnic, lumbar colonic and vagal nerves and of intestinal distension was studied in anesthetized cats. A new flow recording technique was designed. Responses in the ICS to stimulation were recorded as changes in the transsphincteric flow. The response in the ICS was compared to the responses in the intestine proximal and distal to the sphincter. Sympathomimetic amines and autonomic blocking drugs were used in order to elucidate the autonomic mechanism by which the responses to stimulation were mediated.

On the basis of the observations made the following conclusions were drawn

1. Both the splanchnic and the lumbar colonic nerves exert a contractile influence on the ICS. The effect is mediated via excitatory adrenergic α -receptors.
2. The ICS contains inhibitory adrenergic β receptors.
3. Activation of the vagal nerves causes a decrease of the transsphincteric flow which is at least partly due to a direct nervous effect on the sphincteric muscle. A cholinergic mechanism is suggested.
4. Intestinal distension elicits contraction of the ICS. This excitatory intestino-ileo-cecal sphincteric reflex is a spinal reflex and mediated via adrenergic fibres within the sympathetic nervous system acting on α -receptors.

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CONTRACTION AND RELAXATION OF
THE RETRACTOR PENIS MUSCLE AND
THE PENILE ARTERY OF THE BULL

*A study of effects of drugs and transmural nerve
stimulation on isolated smooth muscle strips*

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INTRODUCTION

Erection of the mammalian penis is due to engorgement of the erectile tissue, i.e. the cavernous bodies of the organ. It is generally believed that the primary event in swelling of the erectile tissue is a profound dilation of the arteries forming the inflow channels to the cavernous bodies. This leads to a rapid filling of the erectile tissue because the inflow resistance of the tissue will be lower than the outflow resistance. Cessation of erection is believed to be due to a restoration of the tone of the inflow vessels. Owing to this the inflow resistance of the cavernous bodies exceeds the outflow resistance and the erectile tissue is emptied.

In most subprimate mammals a second and often as important event in erection is a sudden relaxation of the retractor penis muscle. This predominantly smooth muscle is normally continuously contracted and keeps the relaxed penis withdrawn in the prepuce under the skin, hereby protecting the glans penis from damage and contamination with the earth.

Thus, apparently a fundamental mechanism of erection is relaxation of smooth muscle cells, namely those of the arteries supplying the cavernous bodies and those of the retractor penis muscle. On the other hand, a principal mechanism maintaining the penis in the relaxed state is contraction of these smooth muscle cells.

The autonomic nervous pathways responsible for the erection as well as for the maintenance of the resting state of the penis were essentially mapped out during the 19th century by pioneers such as C. Eckhard, Ch. Löwen, W. H. Gaskell, M. François-Franck, J. N. Langley and H. K. Anderson. Their studies showed that the sacral parasympathetic outflow is the main nervous pathway for erection and the lumbar sympathetic outflow the pathway for penile vasoconstriction and contraction of the retractor penis muscle. According to our current concept of autonomic neurotransmission, the neurotransmission of erection would be cholinergic and the neurotransmission of penile vasoconstriction and contraction of the retractor penis muscle adrenergic. However hitherto published studies of this subject have not unanimously pointed to a cholinergic neuromuscular transmission of penile erection. Thus rather conflicting results have been obtained by different authors concerning the action of acetylcholine on the penile vessels and the retractor penis muscle. Further many authors have reported that the erection is resistant to atropine while some investigators have found that erection is suppressed by atropine.

This study deals with the effect of drugs, especially of neurotransmitters, and with the effect of nerve stimulation on isolated smooth muscle strips of the retractor penis muscle and the penile artery of the bull. Special attention has been paid to the question: Is the autonomic neurotransmission of erection cholinergic or not? Our hope was that a study of isolated tissues in a controllable environment might offer some further information on the autonomic

control of the tone of the smooth muscle cells ultimately responsible for penile erection in the mammalian male. In addition to own experiments we have tried to scrutinize the literature available in this field. The effect of adjacent striated muscles on the establishment and maintenance of penile erection is beyond the scope of the present study

CHAPTER I

SURVEY AND SCRUTINY OF EARLIER LITERATURE

At least four review articles have been written which include accounts of the innervation and the pharmacology of the penile vessels and the retractor penis muscle (Müller and Dahl 1912, Gruber 1933 Klinge 1969 a and Bell 1972). However these reviews do not go into details of this subject. Further the two recent articles are rather incomplete concerning work of not English speaking investigators. The survey presented here has deliberately been restricted to studies of the peripheral innervation of the above-mentioned target organs and to the effects of autonomic drugs on these organs.

Historical Survey

The great pioneer in the physiology of penile erection is undoubtedly Eckhard (1863, 1869). It is true that many earlier investigators, as e.g. Müller (1835) and Kölliker (1852) had made several important observations and suggestions. Especially noteworthy is the idea of Kölliker that relaxation of the penile arteries, the smooth muscles of the cavernous bodies and the retractor penis constitutes a most essential condition for erection. Another noteworthy suggestion of Kölliker was that the tone of these smooth muscles was controlled by ganglia at the root of the penis and that the activity of these ganglia was enhanced by the sympathetic system and inhibited by the pelvic nerves. But it was Eckhard who showed that in the dog the main event in penile erection is dilation of the penile arteries. Eckhard (1863) was also the first to demonstrate that penile erection is produced by electric stimulation of the pelvic parasympathetic nerves, which he accordingly called the *nervi erigentes*. He further found that section of the pudic nerves did not produce erection. Eckhard noted that during erection the blood flow in the penile veins is increased and not decreased. Hence it was Eckhard who created the modern concept that the erectile tissue is engorged because the inflow resistance of the cavernous bodies is lower than the outflow resistance. Since then the idea of Eckhard has been confirmed in many studies on dogs including the recent ones of Hart and Kitchell (1966) and Dorr and Brody (1967). This does not exclude that in several mammals events as relaxation of smooth muscle cells within the cavernous bodies, contraction of adjacent striated muscles and certain vasocompression play a role in penile erection (cf Chapter VI). Eckhard (1876) further found that in rabbits, but not in dogs, erection could also be produced by stimulation of the hypogastric nerve.

control of the tone of the smooth muscle cells ultimately responsible for penile erection in the mammalian male. In addition to own experiments we have tried to scrutinize the literature available in this field. The effect of adjacent striated muscles on the establishment and maintenance of penile erection is beyond the scope of the present study

the existence of a synaptic relay in the course of the nerve. Atropine, on the other hand, did not inhibit penile erection evoked by stimulation of the pelvic nerve. The effect of atropine was studied on rabbit which is, like man, devoid of the retractor penis muscle. Spina (1897) using the guinea-pig, also reported that atropine did not prevent penile erection.

Fletcher (1897) working with hedgehog and rat, reported that the excitatory supply (pudic nerve) and the inhibitory supply (pelvic nerve) formed a joint terminal meshwork in the retractor penis muscle. His description is indeed very close to the current concept of the autonomic ground plexus (Hillarp 1945, 1959). Fletcher found no nerve cells in the muscle and according to Fisher (1917) the same applies to the dog retractor penis.

De Zilwa (1901) made an *in vitro* study of the dog retractor penis. Like Sertoli (1883) he found that the smooth muscle showed spontaneous activity and that the tone increased in low but decreased in high temperatures. Electric stimulation of the muscles produced contraction when the muscle was relaxed, but often relaxation if it was in high tone. De Zilwa found that atropine usually relaxed the muscle. Muscarine, on the other hand, produced a slight contraction which could be abolished by atropine. Nicotine relaxed the muscle. De Zilwa further observed that *in vivo* the responses to pudic nerve stimulation were unaffected by atropine and curare.

Elliott (1905) found that in the dog the penile arteries and the retractor penis were contracted by adrenaline. He also found that the muscle showed denervation supersensitivity to this drug. Dale (1906) reported that ergot extracts by themselves caused a slight contraction of the dog retractor penis and abolished contractions induced by adrenaline or by stimulation of the pudic nerve. Thus the motor response of the retractor penis was included already in the two basic studies of adrenergic neurotransmission. In their study of effects of different sympathomimetic amines Barger and Dale (1910) noted only excitatory effects of these compounds on the dog and goat retractor penis but never inhibitory effects despite treatment with ergotoxine.

Fröhlich and Loewi (1906) reported that the effect of pelvic nerve stimulation on the dog retractor penis could be paralyzed with very high concentrations of nitrite.

An early electrophysiological study was performed by Brücke (1910) who found that the contracted dog retractor penis showed rhythmic electrical waves. These waves increased in frequency upon stimulation of the pudic nerve and were abolished by pelvic nerve stimulation (Brücke and Otsuura 1910).

In another of his classic papers Dale (1914) reported that *in vivo* acetylcholine relaxed the retractor penis muscle but *in vitro* the ester had no effect on the muscle. According to Dale the *in vivo* effect was probably due to the drop in blood pressure caused by acetylcholine and not to a direct effect of acetylcholine on the retractor penis muscle since prolonged vagal stimulation also produced a relaxation of the muscle.

Bottazzi (1916) reported that *in vitro* the retractor penis of dog was contracted by histamine tyramine pilocarpine, muscarine and also by atropine but relaxed by papaverine. Edmunds (1930) confirmed that adrenaline contracted the dog retractor penis *in vitro*. This contraction was bolished and even reversed by ergotoxine. The muscle was contracted by pilocarpine. This contraction was abolished by atropine but not by ergotoxine. Atropine by itself caused a slight relaxation. Physostigmine produced a contraction, which was counteracted by atropine. In contrast to De Zilwa's findings Edmunds

found that nicotine contracted the muscle. This effect was also seen after ergotoxine and was therefore assumed to be a direct smooth muscle effect. Edmunds further reported that morphine usually contracted the muscle. Edmunds' paper is the last of the papers in this field published before the concept of chemical neurotransmission was conclusively established. In the following this concept is accepted by the authors and their considerations are influenced thereby.

Henderson and Roepke (1933) using penile perfusion in dogs, found that physostigmine enhanced the effect of pelvic nerve stimulation on venous flow while atropine had almost no effect. Arterial injections of acetylcholine also increased the flow. This effect was abolished by atropine. Henderson and Roepke found no certain amounts of acetylcholine in the perfusion fluid. They concluded that they could not present direct evidence for acetylcholine as neurotransmitter of erection, though the effect of eserine suggested that this was the case.

Bacq (1935) found that in dog eserine greatly enhanced the penile engorgement evoked by pelvic nerve stimulation and that atropine strongly counteracted this engorgement. The vasoconstrictor effect of pudic nerve stimulation was enhanced by cocaine. In agreement with the findings of François-Franck (1895) the sympathetic nerves were found to also carry dilator fibres the effect of which was enhanced by eserine and suppressed by atropine. Bacq stated that the neurotransmission of erection was cholinergic although he was, like Henderson and Roepke, unable to demonstrate any acetylcholine in the penile perfusate. He further explained the lack of effect of atropine found by so many other investigators to have been due to the circumstance that they had not worked with eserinated animals.

Oppenheimer (1938) studied the cat retractor penis *in vivo*. He found that the frequency response curve to sympathetic stimulation as well as the dose-response curve to adrenaline were hyperbolic. Both types of response were enhanced by cocaine. The inhibitory frequency response curve to parasympathetic stimulation was also hyperbolic. He found that acetylcholine duplicated the effect of parasympathetic stimulation. The effect of acetylcholine was enhanced by eserine and abolished by atropine. However atropine did not abolish the effect of parasympathetic stimulation. Oppenheimer stated that the transmitter causing relaxation of the retractor penis was acetylcholine. The resistance to atropine of the parasympathetic response was explained by the concept of Dale and Gaddum (1930) i.e. the muscle cell membranes are in such close proximity to the nerve terminals that their receptors are almost inaccessible to the blocking agent. This theory of Dale and Gaddum called the proximity theory by Ambache (1955) has since then often been adopted in explaining the resistance to atropine of penile erection. Using cats Semans and Langworthy (1938) partially repeated and confirmed the experiments of Langley and Anderson (1895) concerning the origin of the motor and inhibitory nervous supply of the genital organs.

Luduenä (1939) reported that ergotoxine, yohimbine and a piperidine derivative inhibited in the isolated dog retractor penis the response to adrenaline. Further studies (Luduenä 1940) showed that cocaine enhanced the excitatory effect of adrenaline, ephedrine and synephrine, while it reduced that of tyramine and phenylethylamine. Luduenä and coworkers (1949) also found that isoprenaline relaxed the dog retractor penis.

The presence of acetylcholinesterase in the penile erectile bodies of rat and guinea pig was demonstrated by Grieten and Gerehtzoff in 1957. Moderate

doses of atropine have been reported not to impair penile erection in bull (Signoret 1963) rabbit and boar (Dziuk and Norton 1962, Dziuk and Mann 1963) while higher doses may cause insufficient erection in rabbit (Dziuk and Norton 1962). In the study of Baker *et al.* (1964) pilocarpine did not affect the bull's ability to erect the penis.

Orlov (1962, 1963 a and b) studied the autonomic neurotransmission of the dog retractor penis with microelectrodes. He found that the muscle exhibited spontaneous spike activity and also slow waves upon which bursts of spikes could be superimposed. Stimulation of the pudic nerve produced excitatory junction potentials (EJPs) which showed summation and elicited spikes. Adrenaline depolarized the muscle and elicited spikes. Spontaneous miniature EJPs were also seen. These were impaired by sympathetic denervation or reserpine treatment. Repetitive postganglionic stimulation of the pelvic nerve produced an inhibitory junction potential (IJP) and suppressed spontaneous spiking. The IJPs were inhibited by d-tubocurarine and facilitated by physostigmine. Atropine reduced the IJPs in 85 % of the experiments. High concentrations of acetylcholine hyperpolarized the cell membrane and relaxed the muscle. Orlov concluded that the IJP was due to acetylcholine released from the parasympathetic nerves.

Gushchin (1965) studied the effect of adrenaline and acetylcholine on the isolated dog retractor penis. Low concentrations of adrenaline contracted the muscle. These contractions were blocked by dihydroergotamine. Acetylcholine contracted the muscle and these contractions were inhibited by atropine. However when repeated doses of acetylcholine were given the contractile responses became smaller and eventually the response was reversed, i.e. when the acetylcholine concentration had increased the response was inhibition of the muscle tone. Dihydroergotamine had no effect on the acetylcholine contractions. Gushchin assumed that the effect of acetylcholine was dependent on the concentration of the drug and the state of contraction of the muscle. He postulated that parasympathetic nerve stimulation produced very high concentrations of acetylcholine at the neuromuscular junctions and hence the effect of nerve stimulation was relaxation of the muscle. The experiments did not support the idea of a cholinergic release of the adrenergic transmitter.

In a short communication Goldenberg (1965) reported that injected nicotine produced atropine-fast relaxations in the cat retractor penis, which were abolished by hexamethonium. He believed that the effect of nicotine was due to stimulation of parasympathetic nerves but that the liberated transmitter was not acetylcholine.

Luduena and Grigas (1966) made a pharmacological study of the isolated field stimulated dog retractor penis. The response of the untreated muscle was contraction. This was blocked by phentolamine piperoxan bretyllium, guanethidine and local anaesthetics. Bretyllium and guanethidine also produced a gradually developing contraction and then an inhibitory response to nerve stimulation was uncovered. This inhibitory response was not abolished by atropine or scopolamine nor was it enhanced by physostigmine. It was not affected by hemicholinium, hexamethonium or dichloroisoproterenol (DCI) but blocked by propoxycaïne. Acetylcholine contracted the muscle and the contraction was abolished by atropine. Histamine & hydroxytyptamine and nicotine contracted the muscle. The effect of nicotine was abolished by hexamethonium. Luduena and Grigas concluded that field stimulation activated excitatory postganglionic sympathetic adrenergic fibres and inhibitory postganglionic

parasympathetic fibres but that the neurotransmitter of the latter fibres was not acetylcholine. They further found no evidence for a cholinergic link in the adrenergic neurotransmission.

Armitage and Burn (1967) found that in scopolamine-treated dogs acetylcholine contracted the retractor penis muscle. After section of the sympathetic chain this effect was abolished, but then acetylcholine reduced the contractions brought about by injected noradrenaline. They found that eserine enhanced the response to sympathetic stimulation and took these results as support for the theory of a cholinergic link in the adrenergic transmission.

Dorr and Brody (1967) studied the haemodynamics of erection in dogs. They found that stimulation of the pelvic nerves produced a decrease in the arterial resistance of the perfused penis. The effect of pelvic nerve stimulation was abolished by hexamethonium and partly reduced but not abolished, by atropine. However in their study acetylcholine produced no decrease in arterial resistance. No sign of erection was obtained by intra-arterial application of histamine, 5-hydroxytryptamine, bradykinin, DMPP prostaglandin A, noradrenaline, or nitroglycerine. Dorr and Brody found it unlikely that acetylcholine would be directly responsible for the effect of pelvic nerve stimulation but could possibly release a potent vasodilator agent. They considered it likely that the effect of atropine could be due to action on the ganglionic synapse apparently present in the neurogenic pathway.

Hukovic and Bubic (1967) studied the isolated cavernous bodies of the rabbit. The bodies were relaxed by acetylcholine as well as by stimulation of the pelvic nerve. The response to pelvic nerve stimulation was partly reduced by atropine. It was enhanced by physostigmine and hexamethonium. Stimulation of the sympathetic nerves produced contraction this also was diminished by atropine. Noradrenaline and histamine contracted the cavernous bodies. Hukovic and Bubic stressed the importance of cholinergic nerves in the physiological regulation of blood vessel dilation. Working with rabbits Thiesen and coworkers (1969) arrived at the same conclusion as François-Franck (1895) and Bacq (1935) i.e. that also the hypogastric nerve carries erectile fibres although the pelvic nerves constitute the main pathway of erection.

Penttilä and Vartiainen (1964) measured the content of acetylcholine, dopamine, noradrenaline and adrenaline in the cavernous bodies of rabbit and bull. All amines except dopamine were found in rabbit material. The corpus cavernosum penis of the bull lacked acetylcholine and had a very low content of noradrenaline and adrenaline, but like many other tissues from ruminants it contained dopamine. Further studies revealed that the erectile tissues of bull had a very low acetylcholinesterase activity compared with rabbit erectile tissues (Penttilä 1966). Histochemical studies showed that the corpus cavernosum penis of bull lacked adrenergic and cholinesterase-positive nerve terminals, while such terminals were abundant in the erectile tissues of rabbit (Klinge and Penttilä 1969). However adrenergic fibres and slightly acetylcholinesterase-positive fibres have been demonstrated in the penile artery of the bull prior to its entrance into the cavernous bodies (Klinge 1969 b, Klinge and Pohio 1971). Adrenergic nerve terminals have also been observed in the penile vessels and the smooth muscles of the cavernous bodies of cats and macaques (Baumgarten, Falck and Lange 1969). Perfusion studies (Penttilä 1966, Penttilä and Klinge 1966) of bovine erectile tissue showed that adrenaline and noradrenaline increased the inflow resistance. After phentolamine the effect of adrenaline was reversed. Isoprenaline decreased the resistance and this effect was abolished by DCL. Acetylcholine had usually no effect on the resistance, but if

the perfusion pressure was high it decreased the resistance and increased the venous outflow. This effect was blocked by atropine.

Klinge (1970 a) found a high content of noradrenaline in the retractor penis of the bull. The muscle was further reported to contain a relatively large amount of acetylcholine and to exhibit acetylcholinesterase activity (Klinge 1970 b). Histochemical studies revealed an abundance of adrenergic fibres and also acetylcholinesterase-positive fibres (Klinge, Pohio and Solatunturi 1970). There was some distributional correspondence between adrenergic fibres and acetylcholinesterase-positive fibres. Bell and McLean (1970) demonstrated adrenergic fibres along the whole length of the smooth muscle of the dog retractor penis. Acetylcholinesterase-positive nerve terminals were, however almost lacking in the posterior half of the smooth muscle but not in the anterior half. Bell and McLean concluded that the smooth muscle of the dog retractor penis might receive innervation from separate adrenergic and cholinergic fibres. Appreciable levels of acetylcholinesterase did not appear to be associated with adrenergic axons in the dog.

Klinge (1970 c) studied the responses to drugs of the isolated bull retractor penis muscle. It was vigorously contracted by low concentrations of adrenaline and noradrenaline and this effect was abolished by phenoxybenzamine and reversed if the muscle was contracted by bradykinin. The muscle strips were also contracted by histamine and 5-hydroxytryptamine. Acetylcholine in high concentrations irregularly contracted the muscle. Also propionylcholine, butyrylcholine, methacholine, carbamylcholine, bethanechol and nicotine produced contraction when high concentrations were applied. The muscle was further contracted by very low concentrations of bradykinin (Klinge 1969 c). On the base of the lack of relaxing effect of acetylcholine Klinge (1970 b and c, Klinge and Pohio 1971) put forward the hypothesis that the effectors of erection, i.e. the smooth muscle of the penile arteries and the retractor penis, might be supplied by one single type of efferent fibres, i.e. sympathetic adrenergic fibres, and that erection were due to interruption of the sympathetic tone produced by discharges in cholinergic parasympathetic fibres synapsing on the adrenergic neurone. In guinea-pigs treated with reserpine the penis was filled with blood and slightly swollen although there was no actual erection of the organ (Klinge 1972).

In a recent report Luduena and Grigas (1972) studied the effect of various drugs on the dog retractor penis *in vitro*. The muscle was contracted by histamine 5-hydroxytryptamine, adenosine triphosphate (ATP) and prosta glandin F_2 , while γ -aminobutyric acid (GABA) was without effect. But low concentrations of prostaglandin E_1 relaxed the muscle. In referring to their previous study (1968) Luduena and Grigas considered PGE_1 to be the most likely candidate for the role of the parasympathetic transmitter in the dog retractor penis.

Summing Up and Critical Comments

The excitatory innervation of the penile vessels and the retractor penis muscle gives us little problem. It derives from the lumbar sympathetic outflow. Most fibres emanate from the sacral ganglia of the sympathetic chain and reach the target organs via the pudic nerve. Some fibres seem to run in the hypogastric nerve. A few fibres may follow the pelvic parasympathetic nerves. The excitatory neurotransmitter of the sympathetic fibres is

apparently noradrenaline. At least no study presents any doubt in this respect. However here as in other organs there exists a dispute concerning the possibility of a cholinergic link in the adrenergic neurotransmission (Burn and Rand 1965 Burn 1967).

If the excitatory innervation appears to be clarified, this hardly can be said of the inhibitory innervation of the penile vessels and the retractor penis muscle. All investigators agree that the sacral parasympathetic nerves carry inhibitory fibres to the target organs in question. But some authors have also found vasodilator fibres in the sympathetic nerves. This could be the case since erection has been reported to occur in dogs (Müller 1902) and cats (Root and Bard 1947) with severed pelvic nerves when the males mount oestrous females. This points to possible alternative neuronal mechanisms of erection than discharges in the pelvic nerves. When we consider the inhibitory neurotransmission of erection the data available in the literature are indeed both ambiguous and contradictory. Naturally this also applies to the opinions presented by different investigators working in this field. The main question at present is: Is the neurotransmission of erection cholinergic or not? The histochemical and chemical studies indicate that cholinergic fibres are present in the target organs.

We will first consider the information presented on the effects of acetylcholine. Acetylcholine has been reported to produce a decrease in vascular resistance of the dog penis by Henderson and Roepke (1933). But Dorr and Brody (1967) found no such effect. The discrepancy could be due to differences in e.g. perfusion pressure as indicated by the studies of Penttilä (1966) and Penttilä and Klinge (1968). In this context it should be mentioned that Dorr and Brody found no definite effect when studying other well known vasodilators and vasoconstrictors. Further the difficulties in evaluating the exact point of action of a drug when perfusing a vascular bed as that of the penis should not be underestimated. In this context it should be recalled that acetylcholine has been reported to relax the cavernous bodies of the rabbit (Hukovic and Bubic 1967). It seems that the hitherto presented data are inconclusive and emphasize the necessity to examine the effect of acetylcholine on isolated inflow resistance vessels and to compare the effect of the drug with that of stimulation of the inhibitory nerves.

The effect of acetylcholine on the retractor penis muscle may appear still more contradictory. *In vivo* the transmitter relaxes the muscle (Dale 1914, Oppenheimer 1938). Dale ascribed this effect to the fall in blood pressure caused by acetylcholine. This possibility was not considered in Oppenheimer's study in which the blood pressure was not recorded. Therefore Oppenheimer's conclusions of the site of action of acetylcholine are uncertain. Nearly all *in vitro* studies indicate that acetylcholine and other muscarinic agents, if they have any effect, contract the muscle and this effect is blocked by atropine. According to the classic concepts this constitutes the strongest argument against a cholinergic neurotransmission of relaxation of the muscle. However high concentrations of acetylcholine have been reported to relax the muscle (Orlov 1963 b and Gushchin 1965). The mechanism responsible for this effect has not been analyzed. A further discrepancy exists in the literature concerning the effect of nicotine. Most investigators have found a stimulant action of this drug but it has also been found to relax the muscle (De Zilwa 1901). Thus there is a need of reinvestigation of the possible relaxation induced by high concentrations of acetylcholine and by nicotine as well as of the target structures of these drugs before acetylcholine can be discarded as the inhibitory

transmitter of the retractor penis muscle. Further the possibility that acetylcholine can inhibit the excitatory adrenergic neurotransmission has to be investigated.

When the pharmacological studies of the neurotransmission of penile erection are considered, two things are striking 1) All authors who have presented evidence in favour of a cholinergic neurotransmission of erection seem to have used preganglionic nerve stimulation. 2) Most authors, both those presenting evidence in favour of a cholinergic neurotransmission as well as those presenting evidence against this idea, have used very high, without doubt often unphysiological frequencies when stimulating the nerves (e.g. Bacq 100—300 imp/sec, Loduena and Grigas 20 or 60 imp/sec, Dorr and Brody 20 imp/sec, Hukovic and Bubic 60 imp/sec) These two remarks are important. We now know that autonomic ganglia have muscarinic receptors which are stimulated by an excess of acetylcholine and that atropine has ganglionic blocking properties (e.g. Bainbridge and Brown 1960 for further references see Volle and Koelle 1970)

A high frequency of preganglionic nerve stimulation combined as in the experiments of Bacq with physostigmine, is likely to produce stimulation of muscarinic receptors in the ganglia. Hence the suppression by atropine of the effect of pelvic nerve stimulation observed by some authors could entirely be due to a ganglionic effect of the drug. Naturally also the effect of physostigmine reported by many investigators could entirely be of ganglionic origin. However if the studies providing evidence against a cholinergic neurotransmission are taken into consideration, the errors in using a high stimulation frequency are as obvious. Provided the junctional receptors in the target organs are relatively inaccessible to a competitive receptor blocking agent such as atropine (Gaddum and Dale 1930 see also Urtillo and Clark 1956) it might be extremely difficult to record any effect of the blocker if high frequency stimulation yielding an excess of the transmitter in the junctional gaps is applied. Further when the stimulation produces an excess of the transmitter all the receptors may be occupied. Then it might be difficult to record any enhancement produced by a drug preventing the inactivation of the transmitter as physostigmine. This objection could be raised against e.g. the study of Loduena and Grigas (1966). In view of the results of the present study it is a relevant objection because maximum inhibition of the bull retractor penis is obtained with a very limited number of impulses. In this context it also should be mentioned that Armitage and Burn (1967) considered the hemicholinium experiments of Loduena and Grigas inconclusive. According to Armitage and Burn they had too short a period of exposure to hemicholinium (30—150 min). Thus, neither the pharmacological data supporting a cholinergic neurotransmission of erection nor those against it present definite evidence in either direction. The need of experiments with a compound inhibiting the release of acetylcholine, as botulinum toxin, is obvious. There is also a need to determine the frequency response relationship of inhibitory nerve stimulation and to evaluate the effects of various autonomic drugs when the nerves are stimulated with frequencies in a more physiological range, i.e. not exceeding 15 imp/sec.

Finally attention should be paid to two other compounds tentatively suggested to be the parasympathetic neurotransmitter of erection, namely PGE₁ (Loduena and Grigas 1972) and ATP (Bell 1972 in referring to Burnstock

LATERAL VIEW II

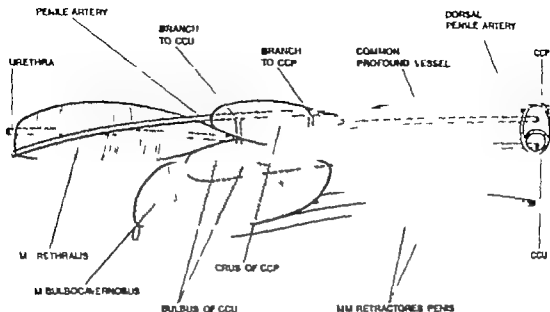


Fig. 2. Schematic drawing of the root of the penis after removal of the ischiocavernosus muscle. ccp = corpus cavernosum penis, ccu = corpus cavernosum urethrae.

artery the vessel reaches the root of the penis on the ventral surface of the pubic bone. In the well protected fat filled space between the pubic bone and the thick tunica albuginea of the crus of the corpus cavernosum penis (ccp) it gives off the branches serving the cavernous bodies (Figs. 2 and 3). The first branch supplies the corpus cavernosum urethrae (ccu) and corresponds to the arteria bulbi urethrae in man. The second branch, which serves the ccp appears to be analogous to the arteria profunda penis in man.

In the bull both branches supplying the cavernous bodies are 1—3 cm long. They are paler and more elastic than the stem of the penile artery. They usually do not ramify before they have entered their respective cavernous bodies. After entrance into the cavernous bodies the branches rapidly lose their thick muscular walls. In the ccu they open directly into the very large sinuoids typical of this body (Grabowski 1937). In the ccp the two branches soon join each other to form a common profound vessel (Fig. 3). The structure of this vessel is quite specific and lacks all the characteristics of an artery. The wall of the vessel is thin and full of small openings resembling a strainer. Thus the arterial supply of the cavernous bodies of the bovine penis considerably differs from that of man and dog, allowing in a simple manner a rapid filling of the erectile tissue. In this connection it should be mentioned that the corpora cavernosa penis of the bull appear to be devoid of autonomic nerves and smooth muscle (Klinge and Penttilä 1969).

After giving off the two branches to the cavernous bodies the penile artery continues as the dorsal penile artery towards the distal parts of the penis parallel with and ventral to the dorsal nerve of the penis.

DORSAL VIEW

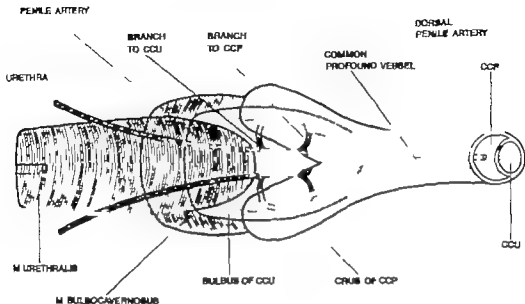


Fig. 2. Schematic drawing of the root of the penis and its arterial supply Abbreviations as in Fig. 2.

(Star Ltd) theophylline ethylenediamine (Medica Ltd) quinidine sulphate (ACO AB), melatonin (Sigma Chemical Company) creatinine hydrochloride (Sigma Chemical Company) strychnine nitrate (Sigma Chemical Company) picrotoxin (Sigma Chemical Company), and yohimbine hydrochloride (Sandoz AG).

Unless otherwise stated all effects of the drugs were verified on at least three preparations. The results of the present report are based on 219 retractor penis strips and 92 penile artery strips obtained from 156 different bulls.

CHAPTER IV

RESULTS

The scope of the present study is outlined in the previous sections. In addition to known and tentative neurotransmitters, as e.g. acetylcholine, noradrenaline, adrenaline, dopamine, 5-hydroxytryptamine, histamine, GABA, glycine, aspartic acid, glutamic acid, Substance P, prostaglandins and ATP also other amino acids and nucleotides have been included. Likewise, in addition to the effects of atropine and other autonomic drugs the effects of certain compounds known to interfere with nervous mechanisms, as e.g. morphine, picrotoxin, quinidine and strychnine, have been studied. Since atropine-resistant parasympathetic effects have been attributed to the release of plasmakinins (Hilton and Lewis 1956, Fülgraff and Schmidt 1963) the effects of bradykinin, kallikrein and aprotinin were studied. Because posterior pituitary hormone preparations have been reported to relax the bull and dog retractor penis (Klinge 1970c, Laiduens and Grigas 1972) and to induce penile engorgement in the dog (Holmquist and Olin 1969) the effects of pure synthetic oxytocin and vasopressin were studied.

The Retractor Penis Muscle

General properties and responses to nerve stimulation

When mounted in the organ bath most preparations exhibited a very low tone during the first 1—3 hours, whereafter the resting tone usually increased and reached a steady level about 2—5 hours after the mounting (Fig. 4). About one fifth of the preparations showed regular spontaneous activity (Figs. 5 and 6). The spontaneous activity could be abolished by increasing the stretch. Spontaneous activity as well as the increase in resting tone seemed to be triggered by field stimulation.

The type of the mechanical response elicited by transmural nerve stimulation was dependent on the stage of contraction, i.e. the resting tone of the muscle strip, and the frequency of the stimuli. During the relaxed stage a contraction was the dominating and characteristic response. When the tone increased the excitatory response proportionally diminished. Simultaneously an inhibitory response immediately following the excitatory one appeared and grew along with the increase in resting tone. The inhibitory response was usually followed by a rather long lasting rebound contraction (Figs. 4

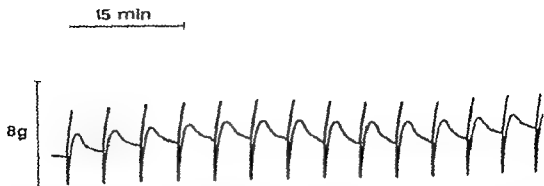


Fig. 4 Retractor penis. Isometric recording. The spontaneous increase in tone is accomplished and a steady state level is reached +5 hours after mounting. Due to the medium stage contraction and to the frequency used the responses to nerve stimulation (3 Hz for 10 sec at 5 min intervals) are typically biphasic. Note also the rebound contraction. Duration of the square wave pulses in this and all the following records is 1 msec.

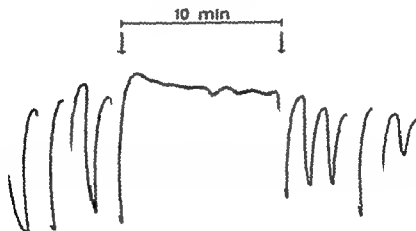


Fig. 5 Retractor penis. Isotonic recording. Load 2.5 g. Magnification 1.4. Spontaneous movements. Continuous field stimulation applied between the arrows with single shocks at 10 sec intervals. The pulse train keeps the strip contracted.

7 19 21 30 and 32 a) The size of this rebound contraction was essentially dependent on the depth of the inhibitory response and the resting tone of the muscle. When the strips were in very high tone there was usually no sign of a rebound contraction. On the other hand, in strips being in low tone the rebound contraction could be very pronounced despite a moderate inhibitory response to nerve stimulation. In these cases the combined response to field stimulation almost had the outlook of a biphasic excitatory response (e.g. Fig. 30). In preparations being in very low tone and where the excitatory response had been blocked by e.g. phentolamine the rebound contraction could be almost the sole visible response induced by field stimulation. As

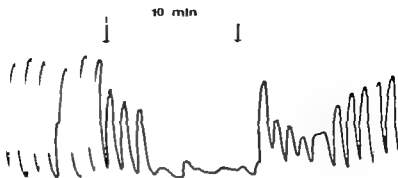


Fig. 6. Retractor penia. Isotonic recording. Same preparation as in Fig. 5 after pre-treatment with phentolamine ($0.7 \mu\text{g/ml}$) which by itself did not interfere with the spontaneous activity. Continuous field stimulation applied between the arrows with single shocks ± 10 sec intervals. The response is reversed and the spontaneous activity is suppressed.

judged from Fig. 7 and from experiments where the excitatory response was suppressed by e.g. phentolamine or enhanced by cocaine (Fig. 19) the rebound contraction was to a certain extent also dependent on the size of the excitatory response.

In sensitive preparations excitatory responses could be evoked by single stimuli (Figs. 8, 22 a and b). Further as seen in the rhythmically contracting

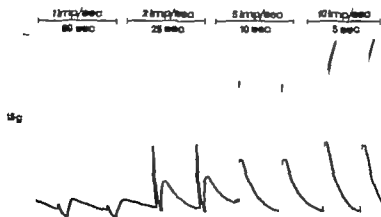


Fig. 7. Retractor penia. Isometric recording. Stimulation with constant number of impulses, i.e. 50, at varying frequencies at 5 min intervals. Note the frequency dependence of the excitatory response.

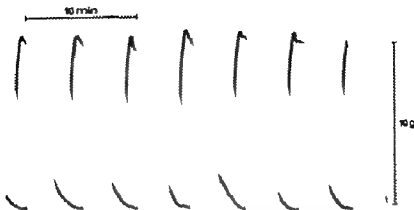


Fig. 8. Retractor penis. Isometric. Responses to single shocks at 5 min intervals. Note the force and duration of the contractions.

preparation in Fig. 5, a long train of impulses delivered at a very low frequency (0.1 imp/sec) could maintain a continuous contraction of the strip. The excitatory response showed a strong dependence on the frequency of stimuli. This is illustrated in Fig. 7 which shows the responses to a constant number of impulses, i.e. to 50 delivered at four different frequencies. A frequency response curve of the excitatory response to our usual 10 sec stimulation is presented in Fig. 9a which shows mean and range of three representative strips from different bulls. As seen in the figure the frequency response curve is fairly flat. However when evaluating this curve it must be kept in mind that the excitatory responses were cut down by the succeeding inhibitory responses which we never were able to selectively abolish or even depress. A true frequency-response curve of the excitation is therefore likely to be considerably steeper. That this might be the case is illustrated in Fig. 9b where the frequency-contraction relationship of a strip taken from the most distal part of the muscle is plotted. This very strip turned out to exhibit almost no inhibitory response to field stimulation. It was, however a rather rigid strip, presumably because of the greater amounts of collagenous tissue in the parts of the muscle close to the insertion. Tissue rigidity as well as paucity of inhibitory nerves might therefore have contributed to the shape of the curve.

The inhibitory response characteristically differed from the excitatory response. This could easily be demonstrated when the preparations were kept in high tone and the excitatory responses were suppressed by an adrenergic neuron blocking agent, as guanethidine, or by an α -receptor blocker as phenoxybenzamine. As illustrated in Fig. 10 the inhibitory response to field stimulation is almost the same at 4, 8 and 16 imp/sec. In Fig. 11 the typical frequency response curve to our usual 10 sec stimulation is presented. The figure gives mean and range of three guanethidine-treated preparations. As seen in the figure the frequency response curve is very steep and maximal inhibition is obtained within 8 imp/sec. This was an almost constant finding. Only rarely was a stimulation frequency higher than 8 imp/sec required to

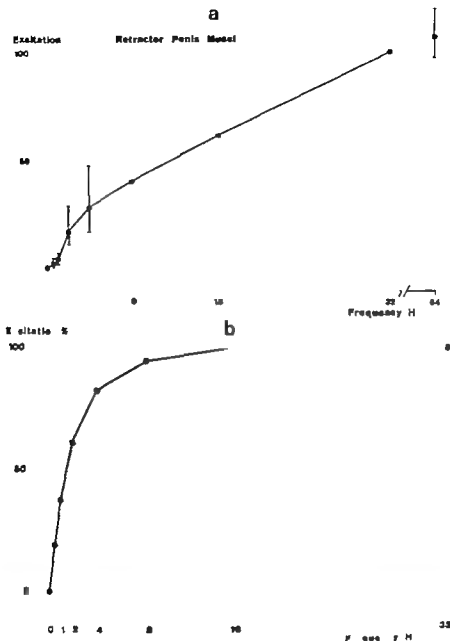


Fig. 9 a. Frequency response curve of isometric contractions to field stimulation. Super maximal voltage, pulse duration 1 msec, train duration 10 sec. The figure gives mean and range of three trips cut from the middle part of the retractor penis muscle from different bulls. The experiments were performed 1–3 h after mounting. The strips were in very low resting tone (0.5–1 g). Here as in the following frequency-response curves the different trains of stimulation were given randomly. Every value from each preparation is the mean of duplicates. The responses are expressed in per cent of the peak response to 32 Hz. Note the fairly flat frequency-response curve. For further comments see the text.

b. Excitatory frequency response curve of preparation cut close to the peripheral insertion of the retractor penis. This preparation proved later (after bretylium) to have almost no inhibitory response to field stimulation. Note the steep frequency-response curve. For further comments see the text.

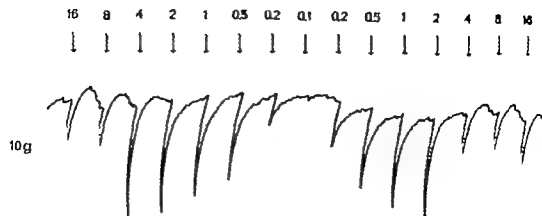


Fig. 10 Retractor penis. Isometric. Pretreatment with bretyllium (6 μ g/ml). Resting tone about 16 g. Stim. for 10 sec at 5 min interval with varying frequencies. Frequency in Hz indicated by numbers above arrows. Note that if the variance in resting tone is taken into consideration the responses to 8 and 16 Hz are identical and the responses to 4 Hz are very close to the responses to 6 and 16 Hz. Cf. Fig. 15.

Inhibition % Retractor Penis Muscle

0

60



Fig. 11 Frequency response curve of isometric relaxation induced by field stimulation. Supramaximal voltage, pulse duration 1 msec, train duration 10 sec. The figure shows mean and range of three strips cut from the middle part of the retractor penis from different bulls. The strips were pretreated with guanethidine (14 μ g/ml) in order to abolish the excitatory response and to maintain the strips in high tone (10-16 g). The responses in this and the two following figures are expressed in per cent of the maximum inhibition obtained with nerve stimulation (the response to 8 Hz for 60 sec). Note the very steep frequency response curve. The slightly reduced response to 32 Hz is probably due to a moderate break through of the excitatory response seen with this very high frequency. For further comments see the text.

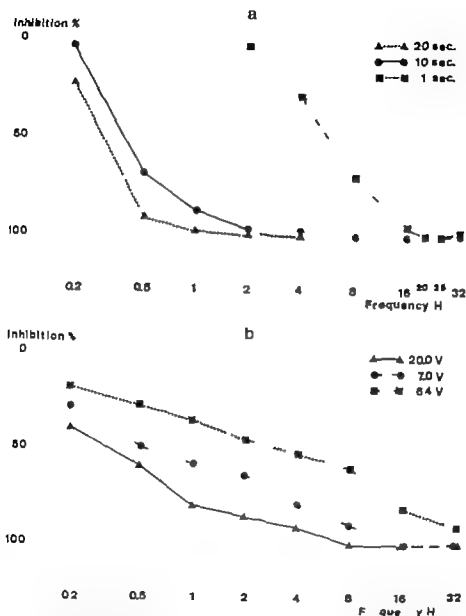


Fig. 12 a. Frequency response curves of inhibitory response to field stimulation of guanethidine-treated ($1.4 \mu\text{g/ml}$) retroractor penis strip. The figure illustrates the effect of changes in duration of the impulse train. Stimulus voltage constant and supramaximal, pulse duration 1 msec. Frequency plotted on a logarithmic scale. An increase in duration of the train to 20 sec, instead of the usual 10 sec, shifts the curve to the left, while decrease of train duration to 1 sec turns the frequency response curve to the right. Note that the same number of impulses, e.g. 2, 4 or 20, causes almost identical degrees of inhibition in the three curves.

b. Frequency-response curves of inhibitory responses to field stimulation of bretylium-treated ($9 \mu\text{g/ml}$) retroractor penis strip. The figure shows the effect of reductions in stimulation voltage, i.e. reduction in number of stimulated nerves. Pulse duration 1 msec, duration of train of stimuli 10 sec. Frequency plotted on logarithmic scale as in a. Reduction in voltage shifts the frequency response curve to the right. With the lowest voltage (6.4 V) maximum inhibition is not reached with the parameters used in the pre-

obtain maximum inhibition. Further if the 8 imp/sec stimulation was extended to periods longer than 10 sec, this usually did not increase the relaxation. In some cases the relaxation rather decreased and stabilized at a slightly higher tone when stimulation was continued (cf Fig 38 b).

If the duration of the impulse train was increased from the usual 10 sec, the frequency response curve was shifted towards the left, i.e. towards lower frequencies, while a shortening of the duration shifted the curve to the right, i.e. towards higher frequencies. This is illustrated in Fig. 12 a where the inhibitory responses to field stimulation at different frequencies and varying durations of the impulse train in a single preparation are plotted on a semi-logarithmic scale. A decrease in stimulation voltage, i.e. a decrease in number of stimulated nerve fibres, also shifted the frequency response curve towards the right (Fig 12 b).

The curve presented in Fig. 12 a also illustrates that with supramaximal stimulation voltage the same total number of impulses produces about the same degree of inhibition. Thus the degree of inhibition to .0 pulses is almost the same when produced by 1 imp/sec for 20 sec, 2 imp/sec for 10 sec or 20 imp/sec for 1 sec. This dependence on the number of the pulses is further illustrated in Figs. 13 a and b. In Fig. 13 a the frequency response curve to 5 10 and 25 impulses delivered at varying frequencies are plotted. The responses are fairly constant in the frequency range of 0.5 to 10 imp/sec. In Fig. 13 b are plotted the impulse response curves from the same preparation at two different frequencies (0.5 and 2 imp/sec). The sigmoid shape of the curves is noticeable. It is also seen that in this preparation the almost maximal inhibition was obtained with about 20 impulses. Thus the inhibitory response to nerve stimulation was characterized by a high dependence on the number of impulses delivered and maximal inhibition was obtained within a limited number of impulses. At frequencies of 0.5 to 10 imp/sec and supramaximal stimulation voltage maximum inhibition was as a rule obtained with 80 impulses or less.

In this connection it should be emphasized that the maximal inhibition obtained by nerve stimulation was not identical with the maximal relaxation of the preparation. The preparations were usually more relaxed when freshly mounted in the organ bath, and when kept in a high tone for study of the inhibitory responses to nerve stimulation further relaxation could always be brought about by e.g. papaverine or theophyllamine. The inhibitory response to nerve stimulation also involved suppression of the rhythmical spontaneous activity as illustrated in Fig 6. A comparison between Fig 6 and Fig. 5 also shows that when continuous stimulation was performed on untreated preparations the excitatory response dominated. This was usually the case in

scanned graph. However it could be reached if the duration of the train was increased. Thus with 8 Hz maximum inhibition was obtained in about 90 sec when stimulation voltage was 0.4 V.

A difference in shape of the frequency response curves to supramaximal voltage and 10 sec duration of train of stimuli can be seen when a and b are compared (middle curve in a, lowest curve in b). In a the response to 0.2 Hz, i.e. to 2 pulses, is relatively smaller than in b. On the other hand, the curve in b is more flat in the higher frequency range, i.e. between 1-8 Hz (10-80 pulses). This difference is probably to a great extent due to the higher resting tone of the strip in b (about 21 g) compared with that of the strip in a (about 11 g). It was a constant finding in this study that responses to very few pulses were much easier to demonstrate in preparations being in very high tone, than in preparations being in low tone.

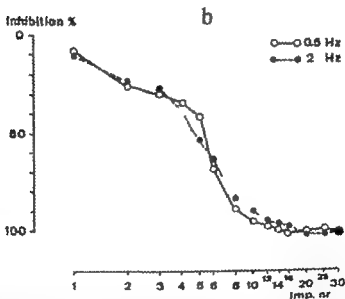
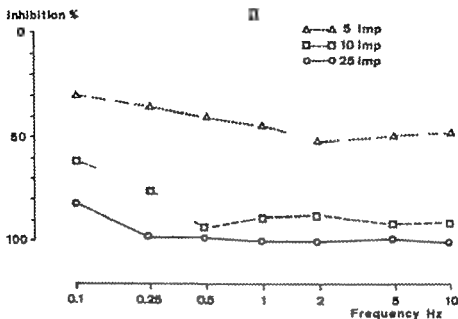


Fig. 13. Inhibitory responses of a retractor penis strip to a fixed number of pulses. The preparation had been treated with phenoxybenzamine ($10 \mu\text{g/ml}$) for 1 h and thereafter washed repeatedly. A constant high tone (23–25 g) was secured by addition of relatively low concentration of barium chloride ($3 \mu\text{g/ml}$).

a. The responses to 5, 10 and 25 pulses delivered at frequencies between 0.1 and 10 Hz are illustrated. Frequency plotted on logarithmic scale. Note that between 0.5 and 10 Hz the response to a certain number of shocks is almost constant regardless of the interval between the individual pulses.

b. Two impulse-response curves of the same preparation as demonstrated. The number of pulses is plotted on logarithmic scale. The shocks were delivered at two different rates, i.e. 0.5 and 2 Hz. Note the sigmoid shape of the two curves which almost coincide.

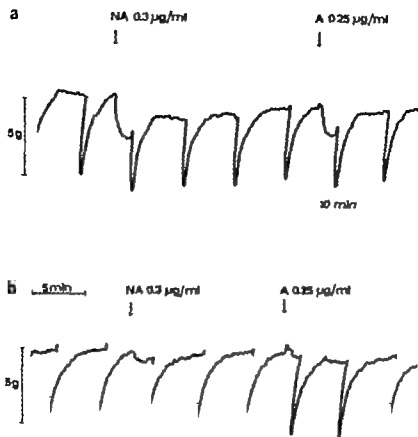


Fig. 16. Retractor penis. Isometric. Pretreated with barium chloride (50 $\mu\text{g/ml}$) and phentolamine (1.5 $\mu\text{g/ml}$). Resting tone about 8 g. Stimulation with 0.5 Hz for 10 sec at 5 min intervals. In a the preparation is relaxed by noradrenaline (NA) and adrenaline (A). In b noradrenaline and adrenaline are almost without effect after addition of propranolol (0.5 $\mu\text{g/ml}$).

was not stimulated. After this the blockade was irreversible but contractions could still be elicited with frequencies exceeding 20–30 imp/sec. Exposure to 100 ng/ml of phenoxybenzamine for one hour resulted in a decrease by 60–80 per cent of the initial excitatory response. With lower concentrations (50 ng/ml) the usual effect was an immediate suppression of the excitatory response to nerve stimulation, which started to grow again if intermittent stimulation was continued. High concentrations of phenoxybenzamine (1–10 $\mu\text{g/ml}$) and phentolamine (4–10 $\mu\text{g/ml}$) usually increased the tone, particularly in field stimulated preparations. Neither of the two α -receptor blocking agents decreased the inhibitory response to nerve stimulation, but due to their effect on the excitatory response and the tone they indirectly increased it.

Propranolol in concentrations up to 2 $\mu\text{g/ml}$ had no distinct effect on the tone of the strip. The responses to nerve stimulation were slightly decreased at very low frequencies (0.1–0.2 imp/sec) but not at higher frequencies (Fig

16b). Two other β -receptor blocking agents with less pronounced local anaesthetic activity practolol (2 $\mu\text{g/ml}$) and sotalol (2 $\mu\text{g/ml}$) were without effect on the responses to nerve stimulation and the tone.

Adrenergic neuron blocking agents and other compounds influencing adrenergic mechanisms

Bretylum (3–12 $\mu\text{g/ml}$) and guanethidine (0.7–4.0 $\mu\text{g/ml}$) effectively blocked the excitatory response to nerve stimulation at frequencies up to 15 imp/sec. Both agents also produced a considerable increase in the tone of the strips and thereby unmasked the inhibitory response (cf Figs. 17, 31, 35, 36 and 37). This increase in tone was accelerated by cocaine (100 ng/ml) and

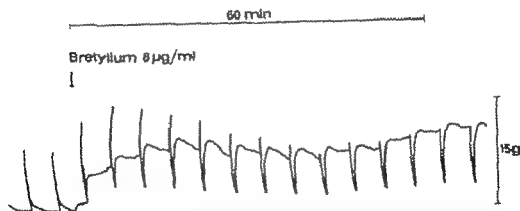


FIG. 17 Retractor penis. Isometric. Stimulation with 3 Hz for 10 sec at 5 min intervals. Bretylum contracts the muscle and initially enhances but subsequently abolishes the excitatory response. The inhibitory response is unmasked (cf Figs. 27 and 45).

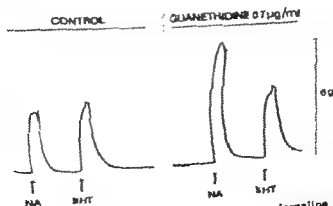


FIG. 18 Retractor penis. Isometric. Contractions to noradrenaline (NA, 100 ng/ml for 30 sec) and 5-hydroxytryptamine (5HT, 100 ng/ml for 30 sec) in the presence of guanethidine (right panel) the effect of noradrenaline is enhanced. This preparation is exceptionally sensitive to 5HT.

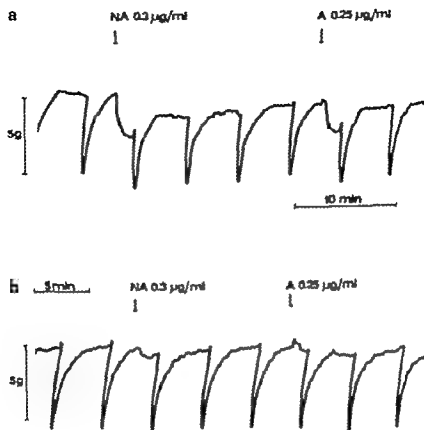


Fig. 16 Retractor penis. Isometric. Pretreated with barium chloride (50 ng/ml) and phentolamine (1.5 μg/ml). Resting tone about 8 g. Stimulation with 0.5 Hz for 10 sec at 5 min intervals. In a the preparation is relaxed by noradrenaline (NA) and adrenaline (A). In b noradrenaline and adrenaline are almost without effect after addition of propranolol (0.5 μg/ml).

was not stimulated. After this the blockade was irreversible but contractions could still be elicited with frequencies exceeding 20–30 imp/sec. Exposure to 100 ng/ml of phenoxybenzamine for one hour resulted in a decrease by 60–80 per cent of the initial excitatory response. With lower concentrations (50 ng/ml) the usual effect was an immediate suppression of the excitatory response to nerve stimulation, which started to grow again if intermittent stimulation was continued. High concentrations of phenoxybenzamine (1–10 μg/ml) and phentolamine (4–10 μg/ml) usually increased the tone, particularly in field stimulated preparations. Neither of the two α-receptor blocking agents decreased the inhibitory response to nerve stimulation, but due to their effect on the excitatory response and the tone they indirectly increased it.

Propranolol in concentrations up to 2 μg/ml had no distinct effect on the tone of the strip. The responses to nerve stimulation were slightly decreased at very low frequencies (0.1–0.2 imp/sec) but not at higher frequencies (Fig.

16b). Two other β -receptor blocking agents with less pronounced local anesthetic activity *practolol* (2 $\mu\text{g/ml}$) and *sotalol* (2 $\mu\text{g/ml}$), were without effect on the responses to nerve stimulation and the tone.

Adrenergic neuron blocking agents and other compounds influencing adrenergic mechanisms

Bretylium (3—12 $\mu\text{g/ml}$) and *guanethidine* (0.7—4.0 $\mu\text{g/ml}$) effectively blocked the excitatory response to nerve stimulation at frequencies up to 15 imp/sec. Both agents also produced a considerable increase in the tone of the strips and thereby unmasked the inhibitory response (cf Figs. 17, 31, 35, 36 and 37). This increase in tone was accelerated by cocaine (100 ng/ml) and

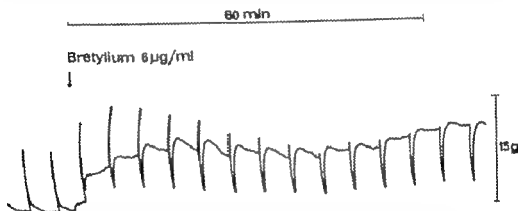


Fig. 17 Retractor penis. Isometric. Stimulation with 2 Hz for 10 sec at 5 min intervals. Bretylium contracts the muscle and initially enhances but subsequently abolishes the excitatory response. The inhibitory response is unmasked. Cf Figs. 27 and 43.

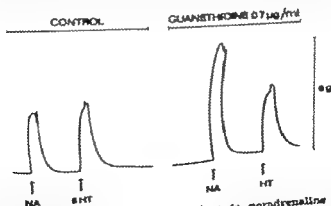
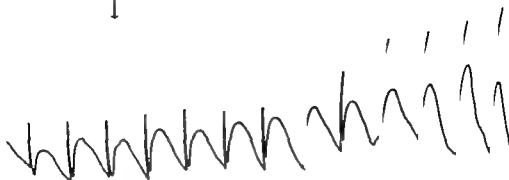


Fig. 18 Retractor penis. Isometric. Contractions to noradrenaline (NA, 100 ng/ml for 90 sec) and 5-hydroxytryptamine (5HT, 100 ng/ml for 90 sec). In the presence of guanethidine (right panel) the effect of noradrenaline is enhanced. This preparation is exceptionally sensitive to 5HT.

Cocaine 5 ng/ml



5g



30 min

Fig. 19 Retractor penis. Isometric. Stimulation with 1 Hz for 10 sec at 5 min intervals. A low concentration of cocaine gradually increases the excitatory response, the rebound contraction and the tone.

could be counteracted by phentolamine ($1.4 \mu\text{g/ml}$). It disappeared promptly after washing, whereas the suppression of the excitatory response was highly resistant to washing. The adrenergic neuron blocking agents enhanced the response to exogenous noradrenaline but not to e.g. exogenous 5-hydroxytryptamine (Fig 18). Because the adrenergic neuron blocking agents suppressed the excitatory response to nerve stimulation and raised the tone of the strips, studies of the inhibitory response and the effects of relaxing compounds were often performed after pretreatment with guanethidine ($1.4 \mu\text{g/ml}$)

Cocaine (5–100 ng/ml) enhanced the excitatory response to nerve stimulation as well as the rebound contraction and raised the tone of the preparations (Fig 19). The onset of the effects was accelerated and their magnitude was augmented by increasing concentrations. Similar effects as those of cocaine were obtained with 10 ng/ml of desipramine whereas 100 ng/ml initially enhanced the excitatory response to field stimulation but later depressed and finally abolished it. However the rebound contraction grew continuously. But also the rebound contraction was suppressed by a still higher concentration ($1 \mu\text{g/ml}$). Cocaine always enhanced the response to exogenous noradrenaline, whereas it was distinctly reduced by $1 \mu\text{g/ml}$ of desipramine. Neither of the two inhibitors of neuronal noradrenaline uptake seemed directly to affect the inhibitory response to nerve stimulation.

a Methyl dopa in concentrations ranging from 0.2 to $20 \mu\text{g/ml}$ did not affect the tone of the muscle or its responses to nerve stimulation. When the concentration was raised to $100 \mu\text{g/ml}$ the excitatory response to field stimulation was slightly reduced after one hour when the usual intermittent stimulation with 10 imp/sec was applied. Nialamide ($6 \mu\text{g/ml}$) had no effect on the tone or on the responses to nerve stimulation during an exposure of 30 min. The other monoamine oxidase inhibitor β -phenyl isopropylhydrazine ($3 \mu\text{g/ml}$) immediately strongly raised the tone of the muscle strip. Presumably this was a direct smooth muscle effect. Pyrogallol ($5 \mu\text{g/ml}$) slightly relaxed the preparation without any obvious effect on the responses to nerve stimulation.

Parasympathomimetic and antimuscarinic agents

Acetylcholine in the concentration range of 1 to 100 ng/ml had no distinct effects on the muscle strips. Higher concentrations (1—100 μ g/ml), however usually had clear actions on the preparations. Three different effects could be distinguished 1) A contraction of the strip which usually lasted 10—15 min, whereafter a moderate relaxation occurred (Figs. 20 and 21). These effects were reduced by atropine (1—10 μ g/ml) 2) An immediate, sometimes complete suppression of the excitatory response to nerve stimulation (Figs. 20, 21 and 22a). This effect lasted at least 20 min and was most prominent when the preparations were stimulated with frequencies lower than 3 imp/sec. Atropine (1—10 μ g/ml) was able to abolish this effect of acetylcholine (Figs. 22a and b). 3) In concentrations of 10—100 μ g/ml acetylcholine produced in some preparations an effect closely resembling that of nerve stimulation, i.e. a brisk contraction immediately followed by a profound relaxation. This effect of acetylcholine also resembled that produced by 10 μ g/ml of nicotine (see below). It was prevented by hexamethonium (100 μ g/ml) but not by atropine.

If the preparations were pretreated with physostigmine the effects of acetylcholine could be obtained with lower concentrations but were qualitatively the same. In preparations contracted by guanethidine 1 ng—100 μ g/ml of acetylcholine had no effect on the inhibitory response induced by nerve stimulation, but in untreated preparations it was indirectly increased due to the transient inhibition of the excitatory response (Fig. 20). Acetylcholine had no overt effect on the responses to exogenous noradrenaline (Fig. 23). The effects of acetylcholine were the same with the chloride and bromide salts. Corresponding concentrations of sodium bromide were without effect.

Pilocarpine was without effect in concentrations ranging from 1 to 100 ng/ml. Higher concentrations (1—100 μ g/ml) slowly raised the tone of the muscle strip. In these concentrations pilocarpine could, like acetylcholine, suppress or abolish the excitatory response to nerve stimulation. This effect of pilocarpine was considerably more long lasting than that of acetylcholine and was also counteracted by atropine (1—2 μ g/ml).

Atropine (1—10 μ g/ml) had no detectable direct effect on the inhibitory response to nerve stimulation but usually relaxed the preparations and then produced a moderate decrease of the excitatory response. Higher concentrations considerably relaxed the strips. Scopolamine exhibited a weaker relaxing effect than atropine and concentrations up to 50 μ g/ml could be added to the bath without causing any conspicuous effect on the tone of the strip. The inhibitory response evoked by the usual stimulation for 10 sec was not influenced by these concentrations of scopolamine. This holds true also for the relaxation produced by continuous stimulation for several minutes.

Ganglionic stimulating and blocking agents

Nicotine in concentrations up to 1 μ g/ml had no effect on the muscle, but 10 μ g/ml produced a rapid transient relaxation (Fig. 24). This was especially prominent in barium-treated preparations (Fig. 25). The relaxation seemed to be facilitated by preceding field stimulation. The speed and slope of the relaxation closely resembled the inhibitory response evoked by field stimulation. But in absence of barium there was no rapid recontraction. The relaxation times preceded by a small but rapid contraction and in those ca

Cocaine 5 ng/ml

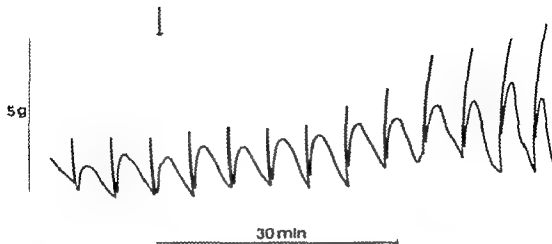


Fig. 19 Retractor penis. Isometric. Stimulation with 1 Hz for 10 sec at 5 min intervals. A low concentration of cocaine gradually increases the excitatory response, the rebound contraction and the tone.

could be counteracted by phentolamine ($1.4 \mu\text{g/ml}$). It disappeared promptly after washing, whereas the suppression of the excitatory response was highly resistant to washing. The adrenergic neuron blocking agents enhanced the response to exogenous noradrenaline but not to e.g. exogenous 5-hydroxytryptamine (Fig. 18). Because the adrenergic neuron blocking agents suppressed the excitatory response to nerve stimulation and raised the tone of the strips, studies of the inhibitory response and the effects of relaxing compounds were often performed after pretreatment with guanethidine ($1.4 \mu\text{g/ml}$).

Cocaine (5–100 ng/ml) enhanced the excitatory response to nerve stimulation as well as the rebound contraction and raised the tone of the preparations (Fig. 19). The onset of the effects was accelerated and their magnitude was augmented by increasing concentrations. Similar effects as those of cocaine were obtained with 10 ng/ml of desipramine whereas 100 ng/ml initially enhanced the excitatory response to field stimulation but later depressed and finally abolished it. However the rebound contraction grew continuously. But also the rebound contraction was suppressed by a still higher concentration ($1 \mu\text{g/ml}$). Cocaine always enhanced the response to exogenous noradrenaline, whereas it was distinctly reduced by $1 \mu\text{g/ml}$ of desipramine. Neither of the two inhibitors of neuronal noradrenaline uptake seemed directly to affect the inhibitory response to nerve stimulation.

Methyldopa in concentrations ranging from 0.3 to $20 \mu\text{g/ml}$ did not affect the tone of the muscle or its responses to nerve stimulation. When the concentration was raised to $100 \mu\text{g/ml}$ the excitatory response to field stimulation was slightly reduced after one hour when the usual intermittent stimulation with 10 imp/sec was applied. Nialamide ($6 \mu\text{g/ml}$) had no effect on the tone or on the responses to nerve stimulation during an exposure of 30 min. The other monoamine oxidase inhibitor β -phenyl-isopropylhydrazine ($5 \mu\text{g/ml}$) immediately strongly raised the tone of the muscle strip. Presumably this was a direct smooth muscle effect. Pyrogallol ($5 \mu\text{g/ml}$) slightly relaxed the preparation without any obvious effect on the responses to nerve stimulation.

Parasympathomimetic and antimuscarinic agents

Acetylcholine in the concentration range of 1 to 100 $\mu\text{g/ml}$ had no distinct effects on the muscle strips. Higher concentrations (1—100 $\mu\text{g/ml}$) however usually had clear actions on the preparations. Three different effects could be distinguished. 1) A contraction of the strip which usually lasted 10—15 min whereafter a moderate relaxation occurred (Figs. 20 and 21). These effects were reduced by atropine (1—10 $\mu\text{g/ml}$). 2) An immediate sometimes complete suppression of the excitatory response to nerve stimulation (Figs. 20, 21 and 22a). This effect lasted at least 20 min and was most prominent when the preparations were stimulated with frequencies lower than 3 imp/sec. Atropine (1—10 $\mu\text{g/ml}$) was able to abolish this effect of acetylcholine (Figs. 22a and b). 3) In concentrations of 10—100 $\mu\text{g/ml}$ acetylcholine produced in some preparations an effect closely resembling that of nerve stimulation, i.e. a brisk contraction immediately followed by a profound relaxation. This effect of acetylcholine also resembled that produced by 10 $\mu\text{g/ml}$ of nicotine (see below). It was prevented by hexamethonium (100 $\mu\text{g/ml}$) but not by atropine.

If the preparations were pretreated with physostigmine the effects of acetylcholine could be obtained with lower concentrations but were qualitatively the same. In preparations contracted by guanethidine 1 ng—100 $\mu\text{g/ml}$ of acetylcholine had no effect on the inhibitory response induced by nerve stimulation, but in untreated preparations it was indirectly increased due to the transient inhibition of the excitatory response (Fig. 20). Acetylcholine had no overt effect on the responses to exogenous noradrenaline (Fig. 23). The effects of acetylcholine were the same with the chloride and bromide salts. Corresponding concentrations of sodium bromide were without effect.

Pilocarpine was without effect in concentrations ranging from 1 to 100 $\mu\text{g/ml}$. Higher concentrations (1—100 $\mu\text{g/ml}$) slowly raised the tone of the muscle strip. In these concentrations pilocarpine could like acetylcholine suppress or abolish the excitatory response to nerve stimulation. This effect of pilocarpine was considerably more long lasting than that of acetylcholine and was also counteracted by atropine (1—2 $\mu\text{g/ml}$).

Atropine (1—10 $\mu\text{g/ml}$) had no detectable direct effect on the inhibitory response to nerve stimulation but usually relaxed the preparations and it produced a moderate decrease of the excitatory response. Higher concentrations considerably relaxed the strips. Scopolamine exhibited a weak relaxant effect than atropine and concentrations up to 20 $\mu\text{g/ml}$ could be added to the bath without causing any conspicuous effect on the tone of the strip. The inhibitory response evoked by the usual stimulation was not enhanced by these concentrations of scopolamine. This holds true also for the relaxation produced by continuous stimulation for several minutes.

Ganglionic stimulating and blocking agents

Nicotine in concentrations up to 1 $\mu\text{g/ml}$ had no effect on the muscle strip. 10 $\mu\text{g/ml}$ produced a rapid transient increase in tone which was most prominent in barium-treated preparations. This effect was usually facilitated by preceding field stimulation. The response closely resembled the inhibitory response to nerve stimulation. In the absence of barium there was no effect on the tone of the strip. 10 times preceded by a small dose of atropine the response was abolished.

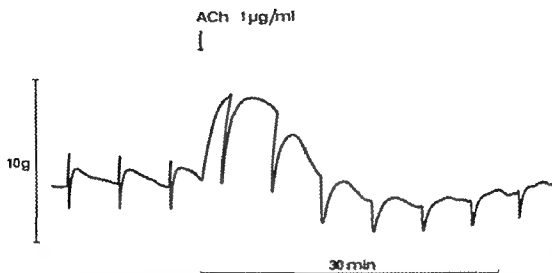


Fig. 20 Retractor penis. Isometric. Stim. 3 Hz for 10 sec at 5 min interval. Pretreated with physostigmine ($1 \mu\text{g/ml}$) Acetylcholine (ACh) contracts the muscle and abolishes the excitatory response. As a consequence of the suppression of the excitatory response the width of the inhibitory response is increased.

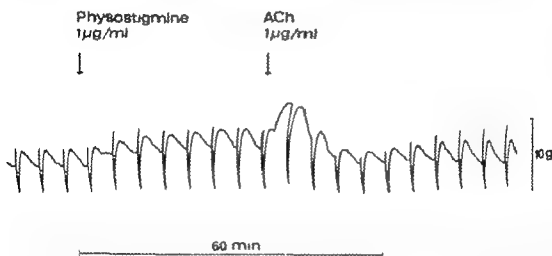


Fig. 21 Retractor penis from castrated bull. Isometric. Stim. 5 Hz for 10 sec at 5 min interval. Physostigmine initially increases the tone and enhances the excitatory response. Acetylcholine (ACh) contracts the muscle and transiently suppresses the excitatory response.

of nicotine mimicked the biphasic response induced by nerve stimulation. The relaxation produced by nicotine was completely prevented by hexamethonium ($100 \mu\text{g/ml}$) mecamylamine ($0.5 \mu\text{g/ml}$) TMA ($5 \mu\text{g/ml}$) McN A 343 ($10 \mu\text{g/ml}$), d tubocurarine ($10 \mu\text{g/ml}$) and lidocaine ($100 \mu\text{g/ml}$). It further was blocked by itself i.e. at least 30 min had to elapse before it could be reproduced. It was only slightly reduced by $1 \mu\text{g/ml}$ of d tubocurarine but was not influenced by atropine ($2 \mu\text{g/ml}$) or pilocarpine ($100 \mu\text{g/ml}$) nor was it affected by a low concentration ($0.8 \mu\text{g/ml}$) of DMPP. It was totally prevented by propranolol

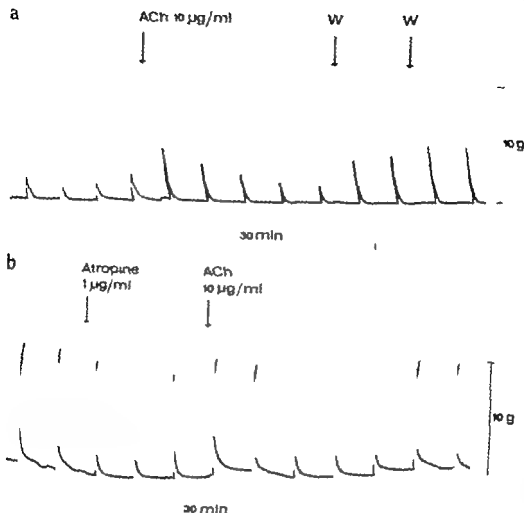


Fig. 22. Retractor penis. Isometric. Stimulated with single shocks at 5 min intervals. In a acetylcholine suppresses the excitatory response. In b after repeated washings, atropine prevents the suppression of the excitatory response by acetylcholine. Cf Figs 46 and 47

($10 \mu\text{g/ml}$), partly reduced by prazosin ($2 \mu\text{g/ml}$), but unaffected by metoprolol ($2 \mu\text{g/ml}$). It was further found that pretreatment with hydrocortisone ($50 \mu\text{g/ml}$), creatinine ($10 \mu\text{g/ml}$), melatonin ($10 \mu\text{g/ml}$) or inosine ($10 \mu\text{g/ml}$) did not affect the relaxation.

In the concentration of $100 \mu\text{g/ml}$ nicotine raised the tone of the muscle and slightly reduced the excitatory responses induced by field stimulation. When the concentration was increased to 1 mg/ml a profound and long lasting relaxation occurred. Washing performed during this relaxation resulted in a rapid and powerful contraction which was almost unaffected by pretreatment with phentolamine ($1.5 \mu\text{g}$) or phenoxybenzamine ($1 \mu\text{g/ml}$) and also by phentolamine ($2 \mu\text{g/ml}$). These effects of nicotine were not prevented by $100 \mu\text{g/ml}$ methonium. The inhibitory responses to field stimulation were not directly influenced by the nicotine concentrations studied.

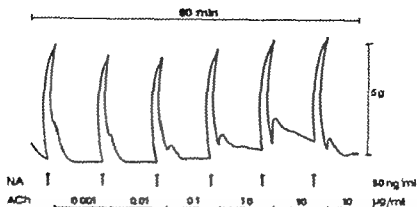


Fig. 23. Retractor penis. Isometric. Contractions produced by the same concentration of noradrenaline (NA, 50 ng/ml for 60 sec) in the presence of increasing concentrations of acetylcholine (ACh). First contraction control. Even in high concentrations acetylcholine is without significant influence on the contractions.

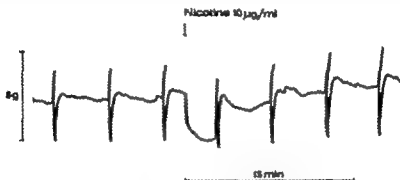


Fig. 24. Retractor penis. Isometric. Resting tone about 5 g. Stim. 10 Hz for 10 sec at 5 min interval. Nicotine (10 µg/ml) induces a rapid relaxation. C/ Figs. 23, 25 and 26.

of nicotine this also applies to the contractions produced by exogenous noradrenaline or 5-hydroxytryptamine.

All the above effects seemed to be produced by nicotine itself since no effects were obtained with the corresponding concentrations of sodium bitartrate.

Also with DMPP (1.2 µg/ml) a brisk response resembling that evoked by nerve stimulation could be obtained (Fig. 26). However the predominant effects of DMPP were a blockade of the excitatory response to field stimulation, an increase in tone of the preparation and an unmasking of the inhibitory response (Fig. 27). These effects always seen with 6 µg/ml closely resembled those produced by guanethidine and bretylium. The rise in tone caused by DMPP was in part prevented by phentolamine (0.75 µg/ml). Like guanethidine DMPP (0.6 µg/ml) potentiated the response to exogenous noradrenaline without influencing the response to exogenous histamine.

The TMA concentration of 0.05 µg/ml was without effect whereas 0.5 µg/ml slightly but distinctly enhanced the excitatory response to field stimulation. This effect was further augmented by 5 µg/ml. The highest concentration used was 50 µg/ml and it moderately raised the tone of the strip and depressed the

Fig. 25. Retractor penis. Isometric. Pretreatment with barium chloride (30 $\mu\text{g}/\text{ml}$). Resting tone about 28 g. Stim. 0.2 Hz for 10 sec at 5 min interval. Nicotine (10 $\mu\text{g}/\text{ml}$) produces a profound but transient relaxation.

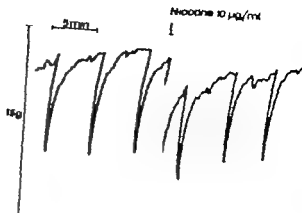
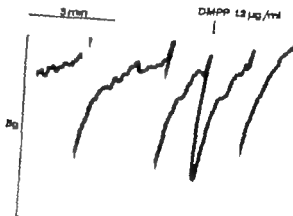


Fig. 26. Retractor penis. Isometric. Pretreatment with barium chloride (30 $\mu\text{g}/\text{ml}$). Resting tone about 18 g. DMPP (1.2 $\mu\text{g}/\text{ml}$) induces a brisk response mimicking those elicited by nerve stimulation (0.8 Hz for 10 sec at 5 min interval.). Cf Figs. 24, 25 and 26.



DMPP $\mu\text{g}/\text{ml}$

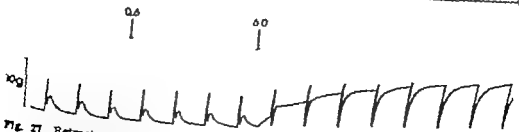


Fig. 27. Retractor penis. Isometric. Stim. 4 Hz for 10 sec at 5 min interval. DMPP (0.8 $\mu\text{g}/\text{ml}$) increases the tone and abolishes the excitatory response. As a consequence of this the inhibitory response is unmasked. Cf Fig. 17.

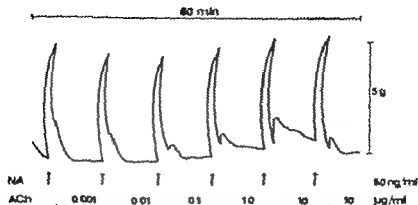


Fig. 23 Retractor penis. Isometric. Contractions produced by the same concentration of noradrenaline (NA, 50 ng/ml for 60 sec) in the presence of increasing concentrations of acetylcholine (ACh). First contraction control. Even in high concentrations acetylcholine is without significant influence on the contractions.

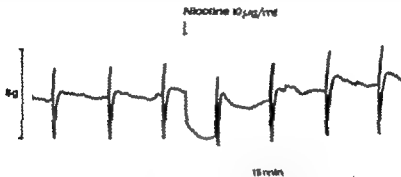


Fig. 24 Retractor penis. Isometric. Resting tone about 6 g. Stim. 10 Hz for 10 sec at 5 min interv. Nicotine (10 µg/ml) induces a rapid relaxation. Cf. Figs. 23, 26 and 42.

of nicotine this also applies to the contractions produced by exogenous noradrenaline or 5-hydroxytryptamine.

All the above effects seemed to be produced by nicotine itself since no effects were obtained with the corresponding concentrations of sodium bitartrate.

Also with DMPP (1.2 µg/ml) a brisk response resembling that evoked by nerve stimulation could be obtained (Fig. 26). However the predominant effects of DMPP were a blockade of the excitatory response to field stimulation, an increase in tone of the preparation, and an unmasking of the inhibitory response (Fig. 27). These effects always seen with 6 µg/ml closely resembled those produced by guanethidine and bretylium. The rise in tone caused by DMPP was in part prevented by phentolamine (0.75 µg/ml). Like guanethidine, DMPP (0.6 µg/ml) potentiated the response to exogenous noradrenaline without influencing the response to *e.g.* exogenous histamine.

The TMA concentration of 0.05 µg/ml was without effect whereas 0.5 µg/ml slightly but distinctly enhanced the excitatory response to field stimulation. This effect was further augmented by 5 µg/ml. The highest concentration used was 50 µg/ml and it moderately raised the tone of the strip and depressed the

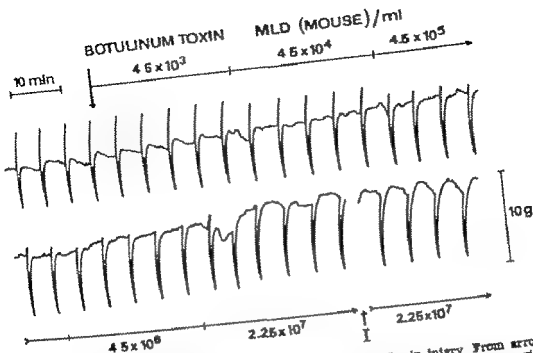


Fig. 22. Retractor penis. Isometric. Stim. 3 Hz for 10 sec at 5 min interval. From arrow on increasing concentrations of botulinum toxin. Upper and lower panel are successive records. I = interval of 25 min. Note the decrease of the excitatory response, while the inhibitory response remains unimpaired.

decrease was seen in the inhibitory response. The decrease of the excitatory response seemed, however not to have been principally due to the hemicholinium *per se* but rather to the intense stimulation, because the same stimulation pattern decreased the excitatory response in untreated preparations too.

Physostigmine (1–10 $\mu\text{g/ml}$) raised the tone of the muscle strips. The inhibitory response evoked by field stimulation was not directly influenced, whereas the excitatory response was in most preparations slightly increased transiently (Fig. 21). The contraction elicited by physostigmine was partly prevented by atropine (2 $\mu\text{g/ml}$) or phentolamine (7.5 $\mu\text{g/ml}$).

Botulinum toxin in increasing concentrations (4.5×10^3 – 2.25×10^7 MLD/ml) did not in the course of more than three hours influence the inhibitory response to nerve stimulation. The higher concentrations of the broth preparation depressed the excitatory response and raised the muscle tone (Fig. 28).

Histamine 5-hydroxytryptamine and agents blocking their receptors

Histamine (0.1–1 $\mu\text{g/ml}$) and 5-hydroxytryptamine (25 ng–1 $\mu\text{g/ml}$) contracted the muscle in a dose-dependent manner without directly affecting the inhibitory response to field stimulation. In some preparations the excitatory response was transiently slightly increased by each compound. Mepyramine relaxed the muscle already in the concentration of 0.07 $\mu\text{g/ml}$. The strips were also slightly relaxed by 1–2 $\mu\text{g/ml}$ of phenazepam, whereas they were contracted by 0.1–1.3 $\mu\text{g/ml}$ of methysergide. None of these three compounds seemed directly to interfere with the relaxation elicited by transverse nerve

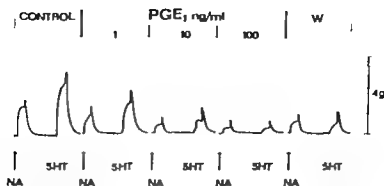


Fig. 29 Retractor penis. Isometric. The contractions produced by noradrenaline (NA, 100 ng/ml for 90 sec) and 5-hydroxytryptamine (5HT, 25 ng/ml for 90 sec) are equally reduced by increasing concentrations of PGE_1 . W = 30 min later after washing. This preparation exhibited exceptionally high sensitivity to 5HT.

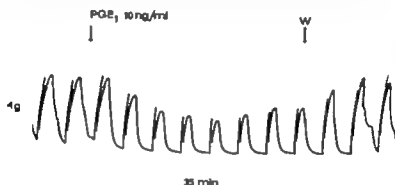


Fig. 30. Retractor penis. Isometric. Stim. 2 Hz for 10 sec at 5 min interval. PGE_1 attenuates the excitatory response and the rebound contraction. W = washing. Cf. Fig. 51.

stimulation. Cyproheptadine (0.2–20 $\mu\text{g/ml}$) relaxed the muscle and depressed the excitatory response to field stimulation but did not directly affect the inhibitory response. In a concentration of 0.2 $\mu\text{g/ml}$ cyproheptadine totally blocked the responses to histamine (1 $\mu\text{g/ml}$) and 5-hydroxytryptamine (1 $\mu\text{g/ml}$) whereas the response to an equipotent concentration of noradrenaline (50 ng/ml) was reduced by about 50 %.

Prostaglandins: prostaglandin antagonists and inhibitors of prostaglandin synthesis

PGE_1 (1–100 ng/ml) produced a clear-cut relaxation in contracted muscle strips. It further decreased in a dose-dependent way the responses to exogenous noradrenaline and e.g. 5-hydroxytryptamine (Fig. 9). The maximal inhibition was achieved with 0.1 $\mu\text{g/ml}$, i.e. a further increase in concentration (1 $\mu\text{g/ml}$) did not produce stronger inhibition. The excitatory responses evoked by field stimulation were also suppressed by PGE_1 (Fig. 30). This could be counteracted in part by doubling the calcium chloride concentration in the organ

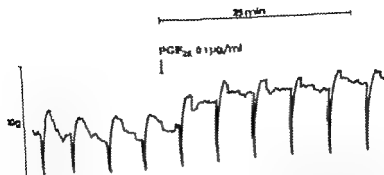


Fig. 31. Retractor penis. Isometric. Pretreated with guanethidine ($1.4 \mu\text{g/ml}$) Stim. 4 Hz for 10 sec at 5 min interval. PGE_{2a} in contrast to PGE_1 , increases the tone of the muscle.

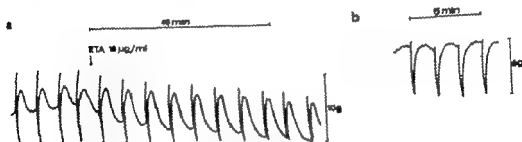


Fig. 32. Retractor penis. Isometric. Stim. 3 Hz for 10 sec at 5 min interval. In a ETA relaxes the muscle strip without overtly influencing the responses to nerve stimulation. In b after 8 hours' exposure to ETA ($10 \mu\text{g/ml}$) the tone of the strip is raised with guanethidine ($1.4 \mu\text{g/ml}$). Note the persistence of the inhibitory responses.

bath. The relaxations produced by PGE_1 were resistant to propranolol ($0.5 \mu\text{g/ml}$), which fully blocked relaxations of identical size induced by isoprenaline. The prostaglandin antagonist SC 19220 ($20 \mu\text{g/ml}$) did not counteract relaxations induced by PGE_1 . PGE_2 ($1-100 \text{ ng/ml}$) had similar but weaker effects than PGE_1 . PGE_2 ($0.1-1 \mu\text{g/ml}$) raised the tone of the muscle (Fig. 31) and slightly enhanced the excitatory response to field stimulation. None of the above prostaglandins appeared to affect directly the inhibitory response to nerve stimulation. SC 19220 ($2-20 \mu\text{g/ml}$) produced a moderate relaxation of the strip and slightly reduced the excitatory response to field stimulation but the inhibitory response was unimpaired.

Of the compounds known to inhibit the synthesis of prostaglandins ETA ($10 \mu\text{g/ml}$) relaxed the muscle without directly affecting the excitatory response elicited by transmural nerve stimulation (Fig. 32 a). Neither did it influence the inhibitory response (Fig. 32 b). Also indomethacin ($1 \mu\text{g/ml}$) was without effect on the responses to nerve stimulation, i.e. the strip stimulated for more than three hours with 5 or 30 imp/sec for 10 min intervals exhibited responses identical with those before application.

Amino acids

The tone of the muscle was slightly decreased and the excitatory response brought about by nerve stimulation was somewhat cut down by 0.1 $\mu\text{g/ml}$ of GABA but these effects were not augmented by 1 $\mu\text{g/ml}$. The concentrations of 10–20 $\mu\text{g/ml}$, on the other hand, increased the tone of the strips. The inhibitory responses were unaffected. Glycine had no effect on the responses to field stimulation but 0.1 $\mu\text{g/ml}$ slightly relaxed the muscle and also here the effect was not rendered greater by 1 $\mu\text{g/ml}$, while 10–20 $\mu\text{g/ml}$ moderately contracted the preparations. The effects of glutamic acid (0.1–20 $\mu\text{g/ml}$) closely resembled those of GABA. Aspartic acid (0.1–20 $\mu\text{g/ml}$) had no effect. When studied in the concentration of 10 $\mu\text{g/ml}$ the following amino acids were without any distinct effect: alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, N-acetyltyrosine, tryptophan, cysteine, methionine, proline, hydroxyproline, glutamine, asparagine, histidine, arginine and lysine.

Nucleosides and nucleotides

Adenosine (10 $\mu\text{g/ml}$) increased the tone and moderately enhanced the excitatory response without influencing the inhibitory response to nerve stimulation. Cytidine, guanosine and inosine had no effect on the preparation in the concentration of 10 $\mu\text{g/ml}$.

ATP (1–10 $\mu\text{g/ml}$) always contracted the muscle strip. This effect was partially and reversibly reduced by quinidine (80 $\mu\text{g/ml}$). It was not abolished by phentolamine (0.75 $\mu\text{g/ml}$). ADP (2–5 $\mu\text{g/ml}$) had similar effects as ATP although they were somewhat weaker. Also AMP (2–20 $\mu\text{g/ml}$) increased the tone of the preparations but its effect was still weaker than that of ADP. The effects of the adenosine nucleotides were characterized by considerable tachyphylaxis. No overt effect on the responses to field stimulation was observed. Cyclic AMP in concentrations ranging from 0.001 to 10 $\mu\text{g/ml}$ had no distinct effect, whereas 100 $\mu\text{g/ml}$ slightly depressed the excitatory response without influencing the tone or the inhibitory response. CTP (0.01–5 $\mu\text{g/ml}$), ITP (0.01–10 $\mu\text{g/ml}$), GTP (0.01–5 $\mu\text{g/ml}$), GDP (5 $\mu\text{g/ml}$) and GMP (5 $\mu\text{g/ml}$) were without any effect on the muscle strips.

Peptides

The strips were contracted by very low concentrations of bradykinin (0.5 ng/ml), eliodisin (10 ng/ml) and Substance P (50 ng/ml) as illustrated in Figs. 33, 34 and 35. These peptides did not affect the inhibitory response to field stimulation but often initially enhanced the excitatory response. Since the solvents of the bradykinin and the eliodisin preparations considerably relaxed the muscle strips it is likely that the retractor penis is contracted by still lower concentrations of these peptides. No definite effect was obtained with pure angiotensin amide until concentrations of 0.05–0.1 mg/ml were reached. Then some preparations were slightly relaxed, but the responses to nerve stimulation were unaffected. With the commercial preparation Hypertensine® moderate relaxations were obtained with lower concentrations of angiotensin amide. They were probably due, however, to the mannitol present in the preparation since mannitol itself relaxed the strips. The more synthetic

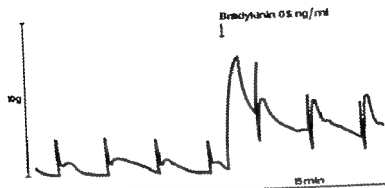


Fig. 33. Retractor penis. Isometric. Stim. 2 Hz for 10 sec at 5 min interval. A low concentration (0.5 ng/ml) of bradykinin contracts the preparation and initially enhances the excitatory response.

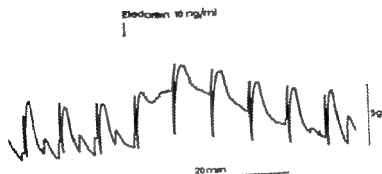


Fig. 34. Retractor penis. Isometric. Stim. 2 Hz for 10 sec at 5 min interval. Also eledone produces a considerable rise in the tone of the muscle.

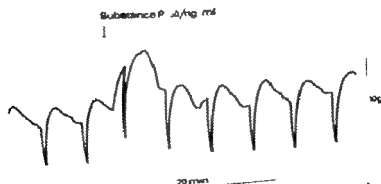


Fig. 35. Retractor penis. Isometric. Pretreatment with guanethidine (14 μ g/ml, 10 min, 4 Hz for 10 sec at 5 min interval). Substance P (50 ng/ml) further increases the tone.

Of other ions the effects of *magnesium* and *potassium* were studied. A increase of the original *magnesium* chloride concentration (100 $\mu\text{g/ml}$) to 400 $\mu\text{g/ml}$ resulted in a rapid and long lasting relaxation. Doubling of the original *potassium* chloride concentration (200 $\mu\text{g/ml}$) produced a rapid and profound but very transient relaxation.

Ergotamine (3 $\mu\text{g/ml}$) vigorously contracted the muscle strips, whereas they could not be relaxed by *adrenaline* (0.5 $\mu\text{g/ml}$) or *isoprenaline* (100 $\mu\text{g/ml}$). In relaxed preparations the inhibitory response induced by field stimulation was unmasked. *Papaverine* (0.1–1 $\mu\text{g/ml}$) profoundly relaxed the muscle and depressed the excitatory response. Also *theophylline* (0.08–8 $\mu\text{g/ml}$) strongly relaxed the muscle and attenuated the excitatory response without directly facilitating the inhibitory response.

Morphine in concentrations ranging from 0.008 to 0.8 $\mu\text{g/ml}$ had no effect on the tone of the strips but it was slightly decreased by still higher concentrations (8–80 $\mu\text{g/ml}$). None of these concentrations affected the responses evoked by nerve stimulation.

Quinidine in a concentration of 0.8 $\mu\text{g/ml}$ slowly suppressed the excitatory response to nerve stimulation. When the concentration was increased to 80 $\mu\text{g/ml}$ the excitatory response was abolished. This effect seemed irreversible since the excitatory response did not reappear despite repeated washing of preparations exposed to a high concentration of *quinidine*. However there was only a slight reduction of the muscle tone and the inhibitory response.

Picrotoxin and *strychnine* in concentrations up to 10 $\mu\text{g/ml}$ exerted no overall influence on either the tone or the responses to field stimulation. *Yohimbine* (1 $\mu\text{g/ml}$) moderately relaxed the strips and slightly decreased the excitatory response to nerve stimulation. These effects became more pronounced with 10 $\mu\text{g/ml}$ but they were never dramatic. No clear effect was seen on the inhibitory response to field stimulation.

The effects of *melatonin* (10 $\mu\text{g/ml}$) and *creatinine* (10 $\mu\text{g/ml}$) were studied in preparations contracted by *barium* chloride (50 $\mu\text{g/ml}$). Neither of these compounds influenced the tone of the muscle or the responses elicited by field stimulation. *Chlorobutanol* and *ethanol* which were present in the solvents of the various commercial peptide preparations relaxed the muscle (cf Fig. 36).

The Penile Artery

General properties and responses to nerve stimulation

When mounted in the organ bath the helical strips of the penile artery usually exhibited a very low tone during the first two hours. Then the tone generally increased rather rapidly and to a relatively greater degree than in the strips of the retractor penis muscle. The spontaneous tone of the arterial strips was rarely as constant as that of the retractor penis preparations. Prominent spontaneous movements were observed in about 10 per cent of the arterial preparations. In common with the retractor penis strips the quality of the response to field stimulation was dependent on the tone of the preparations.

The frequency of the field stimulation had, however less influence on the quality of the responses of the arterial strips. Changes in stimulation frequency mainly altered the magnitude of the responses not their shape. Preparations in low tone showed only excitation and those in a very high tone only inhibition (cf Fig. 54). As in the retractor penis muscle the excitation preceded the inhibition. The speed of both types of response was slower in the arterial preparations than in the retractor penis. This was possibly due to the greater elasticity of the arterial preparations. In general the peak of the excitatory response occurred some seconds after cessation of a 5—10 sec pulse train, whereafter the inhibitory response started to grow. Rebound contractions (e.g. Figs. 51 and 53) following the inhibitory response to field stimulation were rare in the arterial strips. Usually recovery of tone after inhibition was rather slow (e.g. Figs. 44 46 and 50). Only very few arterial preparations responded to single shocks (Fig. 48).

A clear difference between the arterial strips and the retractor penis was revealed when a frequency response analysis was performed. With the usual stimulation for 10 sec both types of response increased up to 64 imp/sec which was the highest frequency used (Figs. 39 and 40). Both graphs give the mean and range of three representative strips cut from the branch serving the ccu (cf Figs. 2 and 3). The frequency analysis of the excitatory response was performed one hour after mounting in the bath. The frequency analysis of the inhibitory response was performed about three hours after mounting, when the preparations were spontaneously in high tone. Guanethidine was added before the examination of the inhibitory response. As seen in the figures, the inhibitory frequency response curve is almost the reverse image of the excitatory frequency response curve. When evaluating the curves it must be kept in mind that the cutting as such implies a change in and also a damage to the tissue. But, under the conditions used the penile artery preparation never showed the same distinct and easily obtained maximum inhibition which was so typical of the retractor penis. In fact, with an increasing number of impulses the inhibitory response increased until the preparation became almost flaccid (Fig. 41).

In essence the above description applies to all the arterial strips used in our study i.e. strips cut from the branches serving the cavernous bodies, the segment between these branches and the segment distal to the branch supplying the ccu. But there were some slight differences. Strips cut from the branches serving the cavernous bodies usually showed quantitatively larger responses than the more rigid strips from the stem of the artery. On the other hand, the latter strips generally exhibited a more stable resting tone. They were therefore preferentially chosen for long term experiments, as for instance when the effects of hemicholinium were studied. A clear difference was noted between arterial strips from young and old bulls. Strips taken from calves always developed considerably less force than those taken from old bulls. However this difference seemed to be entirely quantitative. No qualitative difference in responses to field stimulation was noted between penile arteries from bulls weighing about 200 kg and similar strips from bulls weighing about 1000 kg. Nor was there any qualitative difference between arterial strips from bullocks when compared with strips from uncastrated animals. But strips from castrated bulls were as weak as those obtained from calves. The force developed by an arterial strip seemed roughly to be correlated with the thick-

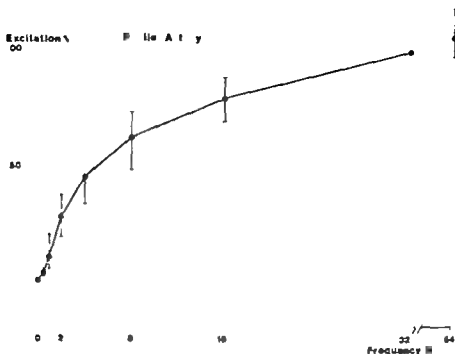


Fig. 39: Frequency response relationship of isometric peak tension developed by helical strips of the penile artery when intramural nerves were stimulated. Supramaximal voltage, pulse duration 1 msec, train duration 10 sec. The figure gives mean and range of three strips cut from the branch to the ccu. All the strips are from different bulls. The responses are expressed in per cent of the response to 32 Hz. Individual values are means of duplicates. The experiments started about 1 h after mounting. The strips were in low tone (0.3–0.4 g).

ness of the arterial wall. Strips which did not spontaneously develop a high tone were treated with barium chloride or ergotamine if the inhibitory responses were to be studied.

In order to make clear that the inhibitory response to transmural nerve stimulation was a specific feature of the penile artery and not a phenomenon shared by all bovine arteries, we examined helical strips from the anterior mesenteric artery the spermatic artery the splenic artery the dorsal metatarsal artery (Fig. 42 versus 43) and branches of the internal pudendal artery serving the distal rectum and the region of the anal orifice and the urinary bladder. We were not able to obtain any sign of an inhibitory response to field stimulation in any of these arteries in spite of treatment with barium chloride and guanethidine. All of them showed an excitatory response which could be blocked by guanethidine. Further when serial segments of the penile artery were analyzed it was found that, although weak, an inhibitory response to field stimulation could be seen in strips taken 4–6 cm, but not more, proximal to the branch supplying the ccu. Inhibitory responses were always obtained in the segments distal to the branch serving the ccu i.e. in the dorsal penile artery. Finally it should be mentioned that strips cut longitudinally from the penile artery exhibited very faint responses to nerve stimulation as well as to drugs.

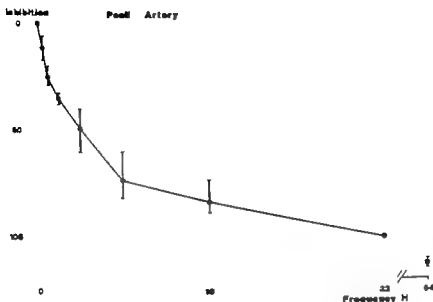


Fig. 40. Frequency response relationship of drop in isometric tension of spiral strips of the penile artery when subjected to transmural nerve stimulation. Supramaximal voltage, pulse duration 1 msec, train duration 10 sec. The figure shows mean and range of three strips taken from the branch to the cca. All the strips are from different specimens. Two strips are identical in this curve and the curve in Fig. 39. As in Figs. 9, 11 and 39, every value from each preparation is the mean of two determinations and the different trains were applied in random order at 5 min intervals. The responses are expressed in per cent of the response to 33 Hz. The experiment started 3–5 h after mounting when the strips had spontaneously developed a high tone (3.5–5 g). Guanethidine (1.4 $\mu\text{g/ml}$) was added shortly before recording started. Note that the inhibitory frequency-response curve in this figure is almost the reversed image of the excitatory frequency response curve in Fig. 39 although somewhat steeper. This circumstance might be explained by the fact that the competing excitatory response was suppressed by guanethidine in the inhibitory frequency response analysis, while nothing could be done in order to suppress the competing inhibitory response in the excitatory frequency response analysis. Note also that 64 Hz produce still greater relaxation than 33 Hz.



Fig. 41. Penile artery segment just proximal to the branch to the cca. Isometric. Pre-treatment with barium chloride (100 $\mu\text{g/ml}$) and guanethidine (1.4 $\mu\text{g/ml}$). Between curves continuous stimulation with 0.5 Hz. Note that the strip continues to relax during the whole stimulation period. Cf Figs. 38 and 39.

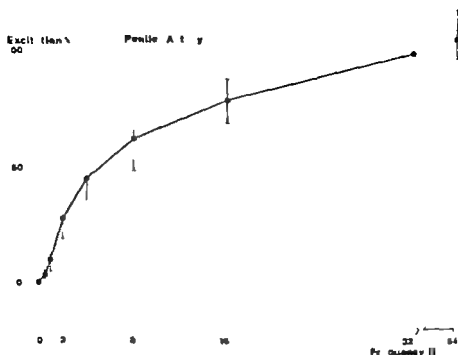


Fig. 39. Frequency response relationship of isometric peak tension developed by helical strips of the penile artery when intramural nerves were stimulated. Supramaximal voltage, pulse duration 1 msec, train duration 10 sec. The figure gives mean and range of three strips cut from the branch to the ccu. All the strips are from different bulls. The responses are expressed in per cent of the response to 32 Hz. Individual values are means of duplicates. The experiments started about 1 h after mounting. The strips were in low tone (0.2–0.4 g).

ness of the arterial wall. Strips which did not spontaneously develop a high tone were treated with barium chloride or ergotamine if the inhibitory responses were to be studied.

In order to make clear that the inhibitory response to transmural nerve stimulation was a specific feature of the penile artery and not a phenomenon shared by all bovine arteries, we examined helical strips from the anterior mesenteric artery the spermatic artery the splenic artery the dorsal metatarsal artery (Fig 42 versus 43) and branches of the internal pudendal artery serving the distal rectum and the region of the anal orifice and the urinary bladder. We were not able to obtain any sign of an inhibitory response to field stimulation in any of these arteries in spite of treatment with barium chloride and guanethidine. All of them showed an excitatory response which could be blocked by guanethidine. Further when serial segments of the penile artery were analyzed it was found that, although weak, an inhibitory response to field stimulation could be seen in strips taken 4–8 cm, but not more, proximal to the branch supplying the ccu. Inhibitory responses were always obtained in the segments distal to the branch serving the ccu i.e. in the dorsal penile artery. Finally it should be mentioned that strips cut longitudinally from the penile artery exhibited very faint responses to nerve stimulation as well as to drugs.



Fig. 44. Penile artery branch to ccu. Isometric. Spontaneously in high tone. Stim. 8 Hz for 10 sec at 3 min interval. Tetrodotoxin obliterates the inhibitory response to field stimulation. Possibly as consequence of this the tone is increased. In this very preparation the inhibitory response to field stimulation appeared to be more sensitive to tetrodotoxin than the excitatory response. Note also the prompt reappearance of the inhibitory response after washing. Cf Fig. 14.

Sympathomimetic amines and adrenergic receptor blocking agents

Adrenaline and noradrenaline (50 ng — 2 μ g/ml) contracted the penile artery in a dose-dependent manner. This effect of the two catecholamines was abolished by phenoxybenzamine (0.1—10 μ g/ml) and phentolamine (0.1—10 μ g/ml). As in the retractor penis, no overt reduction of the inhibitory response to nerve stimulation was seen when the peak of the catecholamine-induced contraction was reached. But before the peak had been reached the inhibitory response to nerve stimulation was usually decreased. A reduction of the inhibitory response to field stimulation was also seen when exceptionally high concentrations (50 μ g/ml) of the catecholamines were used. However in these respects adrenaline and noradrenaline did not differ from other contracting agonists as histamine, vasopressin, acetylcholine or prostaglandins (cf Figs. 45b and 49).

If the arterial strips were pretreated with an α -receptor blocking agent and kept in tone with barium or ergotamine a relaxation could be produced with adrenaline or noradrenaline (0.1—1 μ g/ml). Isoprenaline (0.01—1 μ g/ml) also relaxed contracted preparations and suppressed rhythmic activity in untreated or barium-treated strips. These effects were prevented by propranolol (1—2 μ g/ml).

The excitatory response to field stimulation was inhibited by phenoxybenzamine (1—10 μ g/ml) and phentolamine (1—10 μ g/ml). As in the case of the retractor penis, an effective phenoxybenzamine-induced blockade of the excitatory response elicited by field stimulation required a concentration of 10 μ g/ml and an exposure of one hour during which the preparation was not stimulated. This pretreatment produced an irreversible blockade unless very high frequencies (20—30 imp/sec) were used. When similar treatment was performed with 100 ng/ml of phenoxybenzamine the excitatory responses decreased to about 20—40 per cent of their original height. Because the α -receptor blocking agents usually increased the tone of the preparations and suppressed the excitatory response they unmasked the inhibitory response.

Propranolol (1—5 μ g/ml) and sotalol (1—5 μ g/ml) did not overtly affect the tone of the muscle strips or the response to transmural nerve stimulation.

Adrenergic neuron blocking agents and other compounds influencing adrenergic mechanisms

Bretylium (3–12 $\mu\text{g/ml}$) and guanethidine (0.7–4 $\mu\text{g/ml}$) blocked the excitatory response to nerve stimulation (Fig. 45). Both agents sometimes increased the tone of the arterial preparations, but this effect was never as pronounced as in the retractor penis strips. Neither of them seemed to directly affect the inhibitory response to field stimulation but they uncovered it as a consequence of the suppression of the excitatory response.

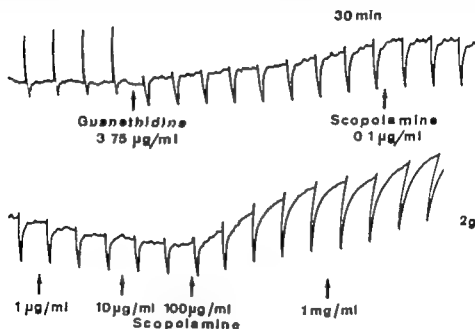


Fig. 45 Penile artery branch to ccp. Pretreated with barium chloride (50 $\mu\text{g/ml}$). Stim. 5 Hz for 5 sec at 5 min interv. Guanethidine blocks the excitatory response and uncovers the inhibitory response. Cf Figs. 17 and 27. Scopolamine in increasing concentrations does not suppress the inhibitory response. High concentrations of scopolamine increase the tone of the strip. Lower panel is a continuous record from the upper curve.

Cocaine (100 ng/ml) enhanced the excitatory response to nerve stimulation and increased the tone of the muscle strips. No clear-cut effect on the inhibitory response was observed.

Parasympathomimetic and antimuscarinic agents

As in the retractor penis muscle three different effects of acetylcholine were seen in the arterial strips: 1) acetylcholine (0.01–100 $\mu\text{g/ml}$) contracted the strips in a dose-dependent way (Figs. 48a and b). The sensitivity to acetylcholine varied considerably between preparations from different bulls but no clear difference was observed between various segments of the penile artery from the same bull. This effect of acetylcholine was blocked by 1–10 $\mu\text{g/ml}$ of

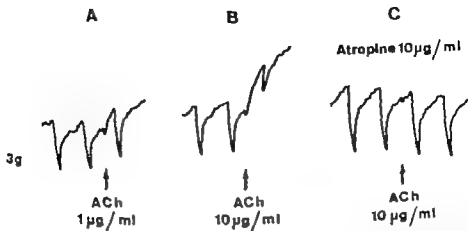


Fig. 46. Penile artery branch to cow. Spontaneously in high tone. Stim. 2 Hz for 10 sec at 3 min interval. A. Moderate contraction to 1 $\mu\text{g/ml}$ of acetylcholine. B. Stronger contraction to 10 $\mu\text{g/ml}$ of acetylcholine. The reduction in size of the inhibitory response probably due to physiological antagonism. C. After atropinization for 20 min, acetylcholine is without effect. Note also that atropine does not reduce the inhibitory response to field stimulation. Cf. Figs. 23 and 47.

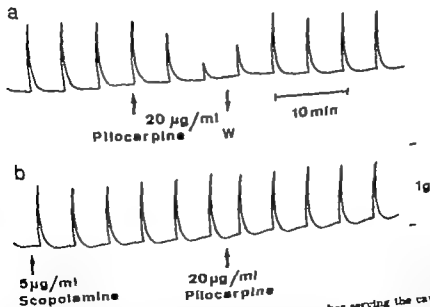


Fig. 47. Penile artery segment of the stem between the branches serving the cavernous bodies. Stim. 6 Hz for 10 sec at 3 min interval. a. Pilocarpine, in a concentration having almost no effect on the tone of the strip, suppresses the excitatory response to field stimulation. W = washing. b. After scopolamine pilocarpine has no effect on the excitatory response. Note also that when the strip is slightly contracted signs of an inhibitory response following the excitatory response become visible. Cf. Figs. 23 and 46.

Adrenergic neuron blocking agents and other compounds influencing adrenergic mechanisms

Bretylium (3—12 $\mu\text{g/ml}$) and *guanethidine* (0.7—4 $\mu\text{g/ml}$) blocked the excitatory response to nerve stimulation (Fig. 45). Both agents sometimes increased the tone of the arterial preparations but this effect was never as pronounced as in the retractor penis strips. Neither of them seemed to directly affect the inhibitory response to field stimulation, but they uncovered it as a consequence of the suppression of the excitatory response.

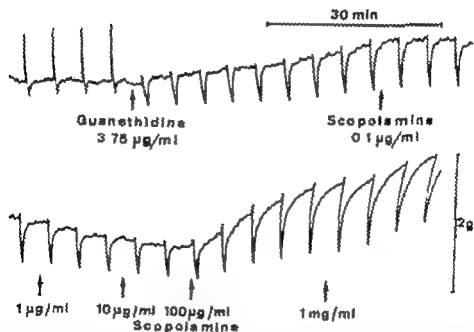


Fig. 45 Penile artery branch to exp. Pretreated with barium chloride (50 $\mu\text{g/ml}$). Stim. 5 Hz for 5 sec at 5 min interval. Guanethidine blocks the excitatory response and uncovers the inhibitory response. Cf Figs. 17 and 37. Scopolamine in increasing concentrations does not suppress the inhibitory response. High concentrations of scopolamine increase the tone of the strip. Lower panel is a continuous record from the upper curve.

Cocaine (100 ng/ml) enhanced the excitatory response to nerve stimulation and increased the tone of the muscle strips. No clear-cut effect on the inhibitory response was observed.

Parasympathomimetic and antimuscarinic agents

As in the retractor penis muscle, three different effects of acetylcholine were seen in the arterial strips: 1) acetylcholine (0.01—100 $\mu\text{g/ml}$) contracted the strips in a dose-dependent way (Figs. 46a and b). The sensitivity to acetylcholine varied considerably between preparations from different bulls but no clear difference was observed between various segments of the penile artery from the same bull. This effect of acetylcholine was blocked by 1—10 $\mu\text{g/ml}$ of



Fig 30. Penile artery branch to ccp. Spontaneously in tone. Stim. 3 Hz for 5 sec at 3 min interv. Physostigmine increases the tone but exerts no certain influence on the inhibitory response to field stimulation.

provided the preparation was in the same stage of contraction as before application of the drug (Fig 49). In arterial strips it was not possible to examine the effect of hemicholinium on the excitatory responses evoked by field stimulation. This was due to the fact that excitatory responses evoked by 30 imp/sec faded considerably within 90 min whether the strips were exposed to hemicholinium or not. Hemicholinium by itself moderately relaxed the strips.

Physostigmine (1–5 $\mu\text{g/ml}$) increased the tone of the arterial preparations. This effect lasted for about 30 min, whereafter the tone slowly returned to the initial level. The magnitude of the inhibitory response was increased concomitantly with the increase in tone. But when the preparation had regained its initial tone the size of the inhibitory response was the same as before the exposure to physostigmine (Fig 50). In relaxed preparations physostigmine produced a moderate increase in the excitatory responses to field stimulation.

Botulinum toxin was used in the concentrations of 4.5×10^5 to 1×10^7 MLD/ml. No distinct effect was seen on the inhibitory response in field stimulation in the course of 5 hours, which was the longest observation period used. Since the experiments were undertaken on strips treated with ergotamine (2–3 $\mu\text{g/ml}$) in order to secure a suitable tone no statement can be made concerning presumptive effects of the toxin on the excitatory response to field stimulation. The toxin preparation relaxed the artery when higher concentrations were added to the bath (4.5×10^6 – 1×10^7 MLD) but recovery of tone soon occurred and the initial tone was regained in about 30 min.

Histamine 5-hydroxytryptamine and agents blocking their receptors

Histamine (0.03–10 $\mu\text{g/ml}$) contracted the artery in a dose-dependent manner. The contractions were abolished by mepyramine (0.1 $\mu\text{g/ml}$) phenbenzamine (0.1 $\mu\text{g/ml}$) and cyproheptadine (0.1–5 $\mu\text{g/ml}$). Also 5-hydroxytryptamine (0.1–10 $\mu\text{g/ml}$) contracted the strips but these contractions exhibited considerable tachyphylaxis. They were inhibited by methysergide (0.1–5 μg) and cyproheptadine (1–5 $\mu\text{g/ml}$). Neither histamine nor 5-hydroxytryptamine seemed to have any direct influence on the inhibitory response elicited by nerve stimulation. The excitatory response was initially increased, i.e. before the preparation had gained a high tone. Mepyramine (1–5 $\mu\text{g/ml}$) and cyproheptadine (1–5 $\mu\text{g/ml}$) relaxed the strips, whereas they were always contracted by methysergide (0.1–5 $\mu\text{g/ml}$). Phenbenzamine (0.1–2 $\mu\text{g/ml}$) had no overt effect on the tone. None of these receptor blocking compounds had any definite effect on the inhibitory response induced by nerve stimulation.

Prostaglandins prostaglandin antagonists and inhibitors of prostaglandin synthesis

PGF₂ (1–100 ng/ml), PGE₁ (0.01–1 µg/ml) and PGE₂ (0.01–1 µg/ml) all contracted the arterial strips in a dose-dependent way (cf Figs 49 and 51). These contractions were counteracted by SC-19220 (10–20 µg/ml) and polyphloretin phosphate (1–10 µg/ml). The excitatory response to nerve stimulation was enhanced by all the three prostaglandins (cf Fig 51). The inhibitory responses increased in size when the preparations contracted (cf Figs 49 and 51). SC 19220 (10–20 µg/ml) reduced the tone of the strips and the excitatory responses brought about by nerve stimulation. In part this was due to the prostaglandin antagonist being dissolved in polyphloretin phosphate. Polyphloretin phosphate in which the compound was dissolved. Polyphloretin phosphate (1–10 µg/ml) also relaxed the strips and suppressed the excitatory response produced by field stimulation. No clear effect was produced by the prostaglandin antagonists on the inhibitory response. Indomethacin (10–20 µg/ml) relaxed the arterial strips. Apparently this was mainly due to the phosphate buffer in which indomethacin was dissolved since the buffer alone also relaxed the strips. The inhibitory responses to nerve stimulation (10 imp/sec) were identical before and after three hours' exposure to indomethacin (10 µg/ml) during which the strips were stimulated with 30 imp/sec for 20 sec every 2 min. This statement is based on judgement of preparations in which the tone was raised with appropriate doses of histamine.

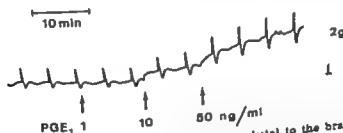


Fig. 51. Penile artery strip from the stem about 4 cm distal to the branch to the corpus cavernosum. Prostaglandin E₁ increases the tone and the excitatory response to field stimulation. The inhibitory response grows with the increase in tone. This strip was from the most distal segment of the penile artery used in the present investigation. Note also the slight rebound contraction following the inhibitory response. Cf Fig 30.

Amino acids

When studied in the concentrations of 1–10 µg/ml the following amino acids were without any definite effect on the arterial strips: GABA, glycine, threonine, phenylalanine, tyrosine, N-acetyltyrosine, tryptophan, cysteine, glutamic acid, aspartic acid, alanine, valine, leucine, isoleucine, serine, methionine, proline, hydroxyproline, glutamine, asparagine, histidine, arginine and lysine.

Nucleosides and nucleotides

Adenosine (3–10 µg/ml) produced a moderate relaxation of the arterial strips. Guanosine (10 µg/ml), cytidine (10 µg/ml) and inosine (10 µg/ml) were without any definite effect.

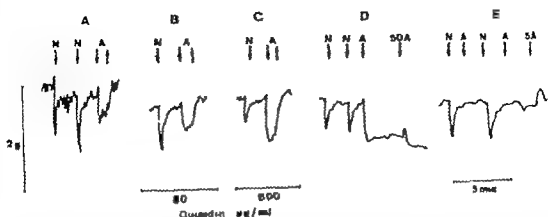


Fig. 52. Penile artery branch to ocp. Spontaneously in tone. At N transmurial nerve stimulation with 2 Hz for 3 sec. At A ATP $10 \mu\text{g/ml}$.
 A. Control record showing relaxations to transmurial nerve stimulation and ATP. B. In the presence of quinidine, $80 \mu\text{g/ml}$ for 10 min, the response to nerve stimulation is reduced but not the response to ATP. C. After quinidine, $800 \mu\text{g/ml}$ for 15 min, the response to nerve stimulation is further reduced, while the response to ATP is increased. D. 10 min after washing out quinidine the responses to nerve stimulation are still reduced and the response to ATP is increased. ATP is not washed out and $500 \mu\text{g/ml}$ of ATP is added at 50A. This high ATP concentration is left in the bath for 30 min. E. After repeated washings the preparation has gained tone. The responses to nerve stimulation are increased, possibly due to recovery from quinidine, but the preparation is now insensitive to $10 \mu\text{g/ml}$ of ATP. However $50 \mu\text{g/ml}$ of ATP (at 5A) contracts the strip. Note also the spontaneous movements in the control record. They are suppressed by quinidine although the drug has no certain effect on the tone of the strip.

ATP ($1-10 \mu\text{g/ml}$) regularly produced a relaxation of the strip (Fig. 52a), sometimes preceded by a brisk contraction. The relaxation was not counteracted but rather enhanced by quinidine in concentrations ($8-800 \mu\text{g/ml}$) which suppressed the responses to nerve stimulation (Figs. 52b and c). However it was easy to desensitize the strips to the relaxing effect of low concentrations of ATP by exposing them to high concentrations of ATP ($0.5-1 \text{ mg/ml}$) for 30 min (Figs. 52d and e). After this treatment low concentrations of ATP had no or even a reversed, effect but the inhibitory response to nerve stimulation was unimpaired (Fig. 52e). ADP ($10 \mu\text{g/ml}$) and AMP ($10 \mu\text{g/ml}$) also produced relaxations but they were considerably smaller than those brought about by ATP.

Cyclic AMP CTP GTP GDP GMP and ITP all in $10 \mu\text{g/ml}$, had no definite effects on the penile artery preparations.

Peptides

No clear-cut effect of bradykinin or elodeisin was seen until the concentration had reached $0.1-2 \text{ g/ml}$. Then the strips relaxed. But this was probably due to the solvent of the peptide preparations since identical relaxations were produced by corresponding concentrations of the solvents alone. Substance P ($0.1-1 \mu\text{g/ml}$) slightly contracted the arterial strips. Neither the excitatory nor the inhibitory response to field stimulation seemed to be directly affected by these peptides.

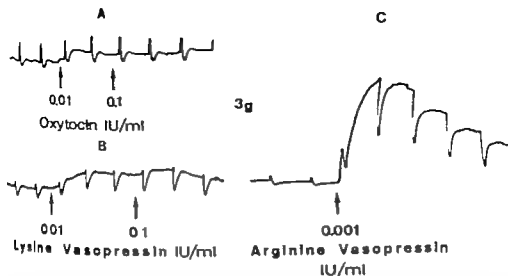


Fig. 43. Penile artery. Three different strips from the same bull. In A branch to corp. B and C branches to corp. Stim. 6 Hz for 8 sec 1.5 min interv. A. Moderate contraction by oxytocin. B. Contraction by lysine vasopressin. C. Strong contraction by arginine vasopressin. Note the marked tachyphylaxis to the hormones shown in A and B and the considerably stronger effect of arginine vasopressin compared with the other hormones. C also illustrates the uncovering of the inhibitory response to field stimulation brought about by a contracting substance. Cf. Fig. 37.

Oxytocin (0.01—1 IU/ml) lysine vasopressin (0.01—1 IU/ml) and especially arginine vasopressin (0.001—0.1 IU/ml) contracted the arterial preparations (Fig. 53). The effects of the posterior pituitary hormones exhibited considerable tachyphylaxis. None of these hormones seemed directly to affect the responses to nerve stimulation.

Angiotensin amide (0.05—1 μ g/ml) contracted the strips, slightly enhanced the excitatory response, and unmasked the inhibitory response to nerve stimulation. The effects of also angiotensin exhibited considerable tachyphylaxis.

Pancreatic kallikrein (0.02—0.2 biological units/ml, Padutin®) raised the tone of the penile artery and induced rhythmic activity in the preparation. Aprotinin substance (1—200 kallikrein inactivating units/ml) on the other hand, relaxed the strip.

Miscellaneous compounds

Bartum chloride (10—100 μ g/ml) raised tone of the strips (Figs. 41 and 43) facilitated the excitatory as well as the inhibitory response to nerve stimulation (Fig. 43) and triggered spontaneous activity. Ergotamine (1—5 μ g/ml) produced a conspicuous contraction of the artery and exposed the inhibitory response evoked by nerve stimulation (Fig. 54).

The arterial strips were markedly relaxed by papaverine (0.1—1 g/ml) and theophylline amine (0.8—8 μ g/ml). These drugs also depressed the excitatory response to field stimulation. Quinidine (8—800 μ g/ml) attenuated both types of response to field stimulation, but particularly the excitatory one. The drug had no unmistakable effect on the tone of the strips but depressed spontaneous rhythmic activity if present. Strychnine (10 μ g/ml) and picrotoxin (10 μ g/ml)

β Adrenoceptors

The present study further confirms the existence of adrenergic β -receptors in the retractor penis (Klinge 1970c) and the penile artery (Penttilä 1966) of the bull. Beta-receptors may also exist in the dog retractor penis (Edmunds 1920 Luduena *et al.* 1949). The physiological significance of the inhibitory β -effect remains obscure particularly as the motor α -effect is so dominant. The present studies with adrenergic blocking agents provide no evidence for any appreciable activation of β -receptors at field stimulation of the intramural nerves. In this connection it should be mentioned that we paid particular attention to the possibility that β -adrenoceptor activation would lead to an increased frequency of spontaneous contractions, i.e. β -receptor activation would have a positive chronotropic effect on the retractor penis and the penile artery as reported for the portal vein (Johansson *et al.* 1967). We never saw any effect of this kind. Isoprenaline always inhibited spontaneous activity. This does, however, not disprove that there could be a positive β -effect on pacemaker activity in the retractor penis and the penile artery. Since β -adrenoceptor activation probably implies hyperpolarization of the smooth muscle cell membranes (cf e.g. Sjöstrand 1973b) it is quite possible that a reduced ability to propagate action potentials would mask any increased activity from pacemaker areas. Particularly if the smooth muscle of the bull retractor penis and penile artery have the same low conduction velocity and ability as the dog retractor penis (Prosser Burnstock and Kahn 1960 Burnstock and Prosser 1960). The possibility therefore remains that locally in pacemaker areas β -adrenoceptor activation could increase the discharge frequency. Finally it is of course not at all necessary to ascribe any physiological significance to β receptors present in organs as the retractor penis and the vas deferens.

Some quaternary ammonium compounds

The close resemblance of some of the effects of DMPP to those of guanethidine and bretylium is in accordance with the concept that this drug also possesses adrenergic neuron blocking properties (Bentley 1962 Birmingham and Wilson 1965 Nedergaard and Bevan 1969).

The rise in tone brought about in the retractor penis and the penile artery by various quaternary ammonium compounds as hexamethonium, TMA and decamethonium may rather be due to a direct muscular effect than to release of e.g. noradrenaline. This assumption is favoured by the fact that their contracting effect was greater on the penile artery than on the retractor penis in contrast to that of bretylium and guanethidine. The TEA induced enhancement of the excitatory response to nerve stimulation is probably due to a facilitated release of the adrenergic neurotransmitter (Thoenen *et al.* 1967 Kirpekar *et al.* 1972).

Local anaesthetics

The increased tone of the retractor penis and the penile artery caused by the local anaesthetics is likely to be a direct smooth muscle effect and not due to release of noradrenaline for the same reasons as the effect of some of the quaternary ammonium compounds, i.e. the effect was usually greater on the

penile artery than on the retractor penis. In the case of procaine the contractile effect was seen only on the artery while the effect on the retractor penis was relaxation.

Hemicholinium botulinum toxin and morphine

Hemicholinium suppresses cholinergic neurotransmission presumably by interfering with the transport of choline to intraneuronal sites (MacIntosh et al. 1956). The onset of the block is known to depend on the concentration of hemicholinium, the frequency of stimuli and the interval between pulse trains (Reitzel and Long 1959). In order to check that the concentration, the time of exposure and the stimulation parameters used were sufficient the following experiments were done. Coaxial stimulation (Paton 1955) of the guinea-pig ileum with 30 imp/sec for 20 sec every 2 min and preganglionic stimulation (Sjöstrand 1962) of the guinea-pig vas deferens (Hukovic 1961) with 20 imp/sec for 10 sec every 2 min were performed in the presence of hemicholinium (80 μ g/ml). Atropine-susceptible responses of the ileum were almost abolished within one hour. The same was the case with the responses to post-preganglionic stimulation of the vas deferens, whereas the responses to post-ganglionic stimulation (Birmingham and Wilson 1963) were almost unaffected. In the retractor penis and the penile artery of the bull no definite reduction of the inhibitory or the excitatory response to field stimulation was observed when preparations exposed to hemicholinium were compared with identically stimulated control strips.

Botulinum toxin type A is regarded to be a specific inhibitor of acetylcholine release from various cholinergic nerve endings (Ambache 1949 Burgen et al. 1949 Ambache 1951). However it has been reported that the toxin can depress adrenergic transmission too although the adrenergic nerve terminals are much more resistant to it (Holman and Spitzer 1973). Thus the reduction in size of the excitatory response seen in this study after botulinum toxin may be due to an impaired release of the transmitter substance from the adrenergic nerve terminals. If this is the case, then the present results indicate that in the retractor penis and the penile artery of the bull the terminals of the inhibitory nerves are still more resistant to the toxin than those of the coxially stimulated nerves. The efficacy of the botulinum toxin used was tested on the coxially stimulated guinea-pig ileum and it was found that 4.5×10^6 MLD (mouse)/ml almost abolished atropine-susceptible responses to nerve stimulation within 45 min.

Morphine was included in the present study because of its inhibitory action on acetylcholine output from cholinergic nerves in the intestine (Schaumann 1957 Paton 1957). Morphine has also been shown to depress the response to adrenergic nerve stimulation in some organs (Szerb 1961 Cairnie et al. 1961) but not in all (Cairnie et al. 1961). In the present study no effect was seen either on the inhibitory or on the excitatory response to field stimulation.

Prostaglandins and prostaglandin receptors

The possible role of the prostaglandins as neurotransmitters will be discussed in the next chapter. In this connection some other aspects will be briefly considered. The prostaglandins E_1 and E_2 have been found to suppress the release of noradrenaline induced by nerve stimulation and hence to diminish motor responses to adrenergic nerve stimulation (Hedqvist and

Brundin 1969 Euler and Hedqvist 1969 for further references see Hedqvist 1973). Because the direct relaxing effect of PGE_1 on the bull retractor penis is so strong, it is impossible to state anything about a presumptive additional contribution of a diminished transmitter secretion when the reduction of the excitatory response to field stimulation after PGE_1 is concerned. The reduction of the excitatory response to nerve stimulation exhibited, however close correlation with the reduction of the motor response to exogenous smooth muscle stimulants.

Since in the penile artery contrary to the retractor penis, the motor response to nerve stimulation was enhanced by all the prostaglandins studied, they probably did not produce a considerable reduction in the release of noradrenaline in that preparation. Maybe the bovine adrenergic neurotransmission is not as sensitive to prostaglandins as e.g. the feline adrenergic neurotransmission. The enhancement of the excitatory response to field stimulation is probably due to the direct stimulant effect of the prostaglandins on the smooth muscle of the artery (cf Sjöstrand 1972).

The prostaglandin antagonist SC-19220 suppressed the excitatory effects of the prostaglandins on the penile artery whereas it did not counteract the inhibitory effect of PGE_1 on the retractor penis. This is compatible with findings in other smooth muscles and further illustrates that prostaglandin receptors mediating relaxation are different from those mediating contraction (Eakins and Sanner 1972).

Smooth muscle stimulants

The reduction in magnitude of the inhibitory response to nerve stimulation frequently seen at the onset of the contraction produced by various stimulating agents, as e.g. barium or noradrenaline, and also when high concentrations of such agents are used, is likely to be due to physiological antagonism. An additive effect due to suppression of the inhibitory neurotransmission in the case of drugs as prostaglandins, α -adrenoceptor stimulants, and muscarinic agents which are known to affect autonomic adrenergic and cholinergic neurotransmission, can of course not be excluded.

The enhancement of the excitatory response often brought about by contracting agents is probably due to a direct synergistic effect on the smooth muscle (Sjöstrand and Swedin 1968 Sjöstrand 1973a).

The effects of barium require some further comments. The general effect of the ion was to raise the tone of the preparations and to facilitate the responses to nerve stimulation. It is known that barium depolarizes smooth muscle and also increases amplitude and duration of action potentials in smooth muscle (see Kuriyama 1970). Barium further facilitates the release of noradrenaline from adrenergic nerve terminals (Kirpekar et al. 1972). Thus the enhancement of the excitatory response to nerve stimulation observed in the present preparations could be the resultant of both neuronal and smooth muscle actions. The facilitation of the inhibitory response to nerve stimulation might as well be the resultant of the raised tone and an increased release of the respective neurotransmitter.

The brief relaxation of the retractor penis observed when the potassium concentration was doubled might be due to excitation of the inhibitory nerves since a more pronounced increase of potassium usually depolarizes smooth muscle and induces contraction.

Quinidine and ATP receptors

Quinidine was tested because this drug has been reported to block the inhibitory effect of ATP on intestines (Bowman and Hall 1970 Burnstock *et al.* 1972) and the excitatory effect of ATP on the urinary bladder (Burnstock *et al.* 1972) and has therefore been used in the analysis of purinergic transmission (Burnstock 1972). In the present study quinidine slightly counteracted the excitatory effect of ATP on the retractor penis while it exerted no antagonistic action at all on the ATP induced relaxation of the penile artery. Burnstock and coworkers (1972) found that quinidine reversed the excitatory effect of ATP on the urinary bladder of the guinea-pig. Thus different receptors for ATP (quinidine-susceptible and not quinidine-susceptible) may occur in smooth muscle (Burnstock 1972). In the present study quinidine inhibited the responses to field stimulation in the retractor penis and the penile artery but in both tissues the excitatory response was considerably more susceptible to this effect than the inhibitory response. This effect of quinidine is probably to a great extent due to the localanaesthetic property of the drug which is characterized by rather long duration.

CHAPTER VI

DISCUSSION

The smooth muscle of the retractor penis and the penile artery

Obviously the bull retractor penis possesses certain properties characteristic of single unit smooth muscle (Bozler 1948). Thus, it can exhibit spontaneous rhythmic activity. This indicates that at least part of the muscle cells have pacemaker activity. The rhythmic contractions, as illustrated in e.g. Fig. 5 have considerable amplitude and duration. This implies that a large muscle area is involved in the contraction and indicates a spread of the activation from cell to cell. Single unit characteristics have been demonstrated with electrophysiological methods in the dog retractor penis (Orlov 1963a). The early findings of Sertoli (1883), De Zilwa (1901) and Edmunds (1920) also point in this direction. The frequency of rhythmic contractions is low. Usually 1 to 3 minutes elapsed between the peaks of each contraction (e.g. Figs. 5 and 6). To some extent this could have been due to the low temperature (35°C) in the organ bath. But this temperature is probably a rather physiological temperature with respect to the extraabdominal parts of the muscle. A rather low conduction velocity of the muscle due to structural arrangement and fibre size of the smooth muscle cells may also contribute to the low frequency of spontaneous contractions. This seems to be the case for the dog retractor penis (Prosser, Burnstock and Kahn 1960; Burnstock and Prosser 1960). The present statement concerning the smooth muscle of the retractor penis which is based on *in vitro* experiments seems to be valid also in *in vivo* conditions. Recently Ashdown and Pearson (1973) observed during surgery on anaesthetized bulls that the tonus of the muscle is not uniform throughout the length of the muscle. Contraction seems to occur at irregular intervals along the length of the muscle at these points the muscle shortens. Presumably also the smooth muscle of the penile artery possesses certain single unit properties as indicated by the spontaneous activity exhibited by several preparations. However the spiral cutting probably deteriorates the muscle. Therefore at present a definite statement is not possible.

Excitatory nerves and motor responses to field stimulation

The excitatory nerves in the bull retractor penis and penile artery activated by field stimulation are apparently adrenergic and the receptors mediating excitation α -adrenoceptors. The contractions evoked by transmural stimulation are abolished or profoundly depressed by adrenergic neuron and α -adrenoceptor

lacking agents and augmented by appropriate concentrations of inhibitors of neuronal noradrenaline uptake. Further the indirectly acting sympathomimetic amine tyramine contracts the retractor penis. Contractions elicited by exogenous noradrenaline are also enhanced by compounds known to inhibit the neuronal uptake of this agonist. Finally the organs have a fairly dense adrenergic innervation (Klinge, Pohio and Solatunturi 1970 Klinge and Pohio 1971) and the retractor penis contains large amounts of the adrenergic neurotransmitter (Klinge 1970a). The reviewed literature (Chapter I) indicates that most of the adrenergic fibres reach the retractor penis and the penile artery via the pudic nerve, but a small portion may run down along the hypogastric or the pelvic nerve.

In this connection it should be noted that the bull retractor penis is highly sensitive to exogenous noradrenaline. This is often not the case with organs having a dense adrenergic innervation, as e.g. the vas deferens (Sjöstrand 1961 1965). High sensitivity to the excitatory transmitter in a densely innervated smooth muscle is obviously an advantage if the organ is intended to be kept in continuous tone, as the retractor penis. Hence *in vivo* the tonic discharge of the excitatory nerves can be very low particularly as the smooth muscle itself exhibits spontaneous activity. The possibility that *in vivo* the bull retractor penis is maintained in continuous contractile activity by a very low impulse frequency in the adrenergic nerves is illustrated by the present *in vitro* results: strong contractions could be produced by single pulses (Figs. 8 and 22) and continuous stimulation at very low frequency (0.1 imp/sec) could keep spontaneously active strips in tone (Fig. 5).

The excitatory frequency response curve to 10 sec stimulation of the retractor penis obtained in this study is however fairly flat (Fig. 9a). To a great extent this is certainly due to the counteracting inhibitory response simultaneously elicited by field stimulation as indicated by the curve presented in Fig. 9b. But it should also be emphasized that even if the maximum excitatory response *in vivo* would require a rather high frequency e.g. 10–15 imp/sec this is not incompatible with the assumption that a low tonic discharge is capable of maintaining the muscle in appropriate contraction. The tensions developed by the tiny retractor penis strips, when stimulated with 16–32 imp/sec, were indeed impressive, i.e. they often exceeded 40 grams. Thus, the feasible maximum force of the whole muscle must be considerable and far exceeds the tension necessary to keep the penis withdrawn in the prepuce.

The excitatory frequency response curve of the penile artery presented in this report may with due reservation for the fact that it is obtained from helically cut and thus injured strips, give certain information on the arterial smooth muscle as a neuro-effector system. However *in vivo* the relevant matter is the resistance to blood flow of the arteries which apart from changes in blood viscosity type of flow and length of the tube varies with the reciprocal of the fourth power of the radius of the vessel. Naturally it is impossible to correctly transfer the present frequency response curve of relative isometric tension of arterial smooth muscle to an *in vivo* frequency response curve of vascular resistance to blood flow. But such a curve would most likely have its maximum at a much lower frequency than the curve presented in this report. Probably a very low vasomotor tone is needed to keep a high resistance in the arteries supplying the cavernous bodies, as is the case in most vascular beds (Folkow 1952, Mellander 1960 Folkow and Jell 1971).

Inhibitory nerves and relaxations induced by field stimulation

The inhibitory response to field stimulation of the retractor penis and the penile artery is obviously of neural origin since it is abolished by tetrodotoxin and local anaesthetics. From the information given by the reviewed literature it seems reasonable to assume that the inhibitory nerves excited by transmural stimulation are mainly postganglionic parasympathetic fibres, although it can not be excluded that some fibres could belong to the sympathetic nervous system. Further from evidence in the literature as well as in the present study it seems reasonable to assume that the inhibitory nerves in the retractor penis and those in the penile artery are of the same kind and that they act by releasing an inhibitory agent.

Our results support in no way the concept that the neurotransmitter which relaxes the smooth muscle responsible for erection is acetylcholine. On the contrary the data speaking against acetylcholine as the inhibitory transmitter of these muscles are numerous. Acetylcholine and pilocarpine contract the retractor penis as well as the penile artery (the relaxations sometimes produced by acetylcholine or nicotine will be discussed below). These contractions are inhibited by atropine and scopolamine. Thus, in the strips of retractor penis and penile artery examined in the present study the muscarinic receptors accessible to exogenous cholinomimetics are excitatory. With reservation for the contraction due to physostigmine it can be stated that this drug does not enhance the inhibitory response to field stimulation. Nor is this response impaired by even extreme concentrations of atropine or scopolamine. The response is not inhibited by ganglionic or neuromuscular blocking agents. This as well as the short lasting and tachyphylactic effect of ganglionic stimulants (see below) makes it unlikely that the smooth muscle cells have inhibitory nicotinic receptors. The vigorous contractions of the retractor penis induced by even minute concentrations of bradykinin and the uncertain effect of this compound on the penile artery as well as the observed effects of kallikrein and aprotinin make it unlikely that acetylcholine could relax the muscles indirectly via kinin release. Finally and most conclusively the inhibitory response is unimpaired by hemicholinium and botulinum toxin. For the above reasons we conclude that the inhibitory responses to field stimulation are not due to release of acetylcholine from inhibitory nerves. In this respect we agree with Luduena and Grigas (1966).

Luduena and Grigas (1972) have suggested that the inhibitory neurotransmission could be prostaglandinergic. We find this unlikely. It is true that PGE₁ relaxes the retractor penis of the bull as it relaxes the retractor penis of the dog (Luduena and Grigas 1972) and the cat (Klinge and Sjöstrand, unpublished). However the inhibitory responses to field stimulation are uninfluenced by inhibitors of prostaglandin synthesis. Further the three common prostaglandins PGE₁, PGE₂ and PGF_{2α} contract the penile artery. This is a strong argument against at least these prostaglandins as the inhibitory neurotransmitter.

It is also unlikely that the inhibitory neurotransmission of the retractor penis and the penile artery is purinergic. ATP, ADP and AMP contract the retractor penis. The penile artery is relaxed by ATP but this effect can easily be abolished or even reversed by desensitization to ATP. However this treatment has no effect on the inhibitory response to field stimulation. The other nucleotides and nucleosides studied were without any distinct effect, but this could have been due to penetration difficulties.

The present study provides no evidence in favour of histamine, 5-hydroxy tryptamine or Substance P as the inhibitory neurotransmitter since all these compounds contract both the retractor penis and the penile artery. Further the inhibitory response to field stimulation is uninfluenced by antihistamines and 5-hydroxytryptamine antagonists. It is also unlikely that GABA, glycine, aspartic acid, glutamic acid or any other of the amino acids studied would be the inhibitory transmitter. As a matter of fact the present study does not give even a hint of the nature of the inhibitory neurotransmitter in the retractor penis and the penile artery of the bull.

Recently Gillespie and coworkers (Gillespie 1972, Gibson and Gillespie 1973, Gillespie and McGrath 1973) have studied the rat anococcygeus muscle as well as that of the cat (Gillespie and McGrath 1974). This muscle exhibits great similarities with the retractor penis with respect to e.g. the responses to transneuronal nerve stimulation and the reaction to adrenergic neuron blocking agents. This is hardly a coincidence. In the bull (Heinemann 1937) as well as in the dog (Langley and Anderson 1895) the anococcygeus muscle has a common origin with the retractor penis. Presumably the two muscles have an identical nervous supply. Of particular interest are the findings that treatment with 6-hydroxydopamine or immunosympathectomy does not interfere with the inhibitory response to field stimulation of the rat anococcygeus (Gibson and Gillespie 1973). Further the excitatory adrenergic innervation and the inhibitory innervation of the rat anococcygeus have different spinal origins (Gillespie and McGrath 1973). Therefore, the inhibitory and the excitatory nerves must constitute separate entities.

Although the nature of the inhibitory neurotransmitter remains unsettled, the present study gives certain information on the responses of the retractor penis and the penile artery to inhibitory nerve stimulation. A characteristic feature of the inhibitory response of the retractor penis is the very steep frequency response curve and the easily obtained maximum inhibition. Since additional relaxation could be obtained by e.g. papaverine the restriction of the inhibition induced by nerve stimulation can not be due to a limited capacity of relaxation. Since shortening of the duration of the impulse train and a decrease in stimulation voltage (i.e. decreased number of activated nerve fibres) both shifted the frequency-response curve to the right, i.e. towards higher frequencies, without changing the degree of maximum inhibition, and further since the inhibitory response was mainly dependent on the number of impulses and not on their frequency it is hardly a limited capacity of transmitter secretion from the nerves which restricts the inhibitory response. This is also evident when the frequency response curve of the retractor penis and that of the penile artery are compared. Thus, since it obviously is neither the capacity of relaxation of the smooth muscle nor the limited maximum secretion of the inhibitory nerves which is responsible for the limited inhibition of the retractor penis, it must be an event between transmitter release and relaxation which restricts the inhibition produced by nerve stimulation. A plausible explanation is that the number of receptors for the inhibitory transmitter is relatively limited and that a high degree of receptor occupancy is quickly established. An alternative explanation would be that one of the reactions following occupation of the inhibitory transmitter may be promptly achieved. The rapid and reproducible relaxations in response to nerve stimulation as well as the almost instantaneous cessation of the relaxation when stimulation is interrupted indicate that the sites of transmitter

release and elimination are located close to the receptors in the retractor penis. Probably the retractor penis has a rather dense inhibitory innervation. The high dependence of the inhibitory response on the number of impulses, regardless of the frequency at which they are applied, suggests that the secretion of the inhibitory transmitter shows little facilitation when the interval between the pulses is shortened. With reservation for the fact that the arterial preparation of this study certainly is rather injured, it seems that it has not as intimate a relationship between the inhibitory nerves and the receptors as the retractor penis.

Further speculations on the nature and the receptors of the transmitter of the inhibitory nerves would be premature. The functional implications of the present findings may be considered, however. As an autonomic neuro-effector unit the bull retractor penis with its inhibitory nerves must be regarded as a very efficient system. In almost every preparation a total number of 80 impulses delivered at frequencies ranging from 0.5 to 10 Hz produced maximum inhibition. Usually 10–20 impulses yielded 80–90 % inhibition, and 50 % inhibition or more was often obtained with only 5 impulses. This implies that *in vivo* the retractor penis could relax instantaneously on even moderate discharges in the inhibitory nerves.

The inhibitory frequency response relationship of the arterial strip was very similar to that of the excitation. Possibly *in vivo* the frequency response relationship between the inhibitory discharge and the vascular resistance would also be an almost reversed image of the corresponding excitatory relationship when dilation of constricted arteries is considered. The fundamental point, as evident from the reviewed literature, is that penile erection can not be brought about solely by interruption of the adrenergic vasomotor tone. Vasodilator innervation of the resistance vessels is apparently needed in this vascular bed. An obvious reason for this would be that, at least in the bull (see the anatomical outline) there is hardly any possibility for formation and action of local vasodilator metabolites as, e.g., in muscular and glandular tissues.

The rebound contraction

The often long lasting rebound contraction observed in many preparations from the retractor penis when stimulation of the inhibitory nerves was terminated, is likely to be a true rebound effect similar to that described for the gut (Furness 1970 1971). The marked dependence of the rebound contraction on the degree of the inhibitory stimulation and on the tone of the muscle makes it unlikely that this secondary contraction (Furness 1971) is due to a transmitter release from a third type of nerve. Since moderate concentrations of phentolamine slightly diminished the rebound contraction and cocaine enhanced it, noradrenaline remaining at the receptor sites may to a small extent contribute to the size of the contraction. Rebound contraction of the retractor penis may be of physiological significance by facilitating a rapid termination of penile erection.

Acetylcholine and the role of possible cholinergic innervation

As stated above, it is unlikely that acetylcholine is the inhibitory transmitter responsible for the relaxation caused by transmural nerve stimulation.

However, acetylcholine is present in the bull retractor penis in concentrations corresponding to those of the bovine duodenum and urinary bladder and the tissue also contains acetylcholinesterase (Klinge 1970b). Histochemical studies have shown that the acetylcholinesterase activity is located in nerves scattered in the muscle (Klinge, Pohto and Solatunturi 1970). These authors found no correspondence between the location of the cholinesterase-positive fibres and the adrenergic fibres. In the dog retractor penis Bell and McLean (1970) found a rich distribution of adrenergic terminals along the whole length of the smooth muscle, while acetylcholinesterase-positive fibres were mainly located in the anterior part of the muscle. These authors concluded that the dog retractor penis probably was supplied by separate adrenergic and cholinergic nerves. Accordingly in the retractor penis of the bull, the cholinesterase-positive nerves may at least in part be separate from the adrenergic nerves. It is commonly assumed that acetylcholine present in tissues possessing cholinesterase-positive nerves is located in these nerves. If the above assumptions are valid the bull retractor penis would, in addition to adrenergic nerves, also be supplied by cholinergic nerves, parasympathetic or maybe sympathetic in origin. We will therefore discuss the observed effects of acetylcholine and of other muscarinic and nicotinic drugs on the presumption that the bull retractor penis is innervated by separate cholinergic nerves and we will consider the possible function of such presumptive nerves.

Two different muscarinic actions were observed 1) Contraction of the smooth muscle, and 2) suppression of the excitatory response to field stimulation. Presumptive cholinergic nerves might therefore constitute a component of the excitatory innervation of the muscle and/or contribute to its relaxation by suppressing adrenergic neurotransmission. The first alternative appears less probable although it can not be excluded. The pharmacological analysis of the excitatory response to nerve stimulation provides no clear evidence for an appreciable cholinergic component in this response. Further, many preparations were virtually insensitive to the contracting effect of high concentrations of acetylcholine. Low concentrations, i.e. less than 1 μ g/ml, rarely contracted the strips. The second alternative seems more probable. In some preparations the sole effect of pilocarpine or acetylcholine was suppression of the excitatory response to field stimulation. Inhibition of cholinergic adrenergic transmitter by muscarinic agents in many organs (e.g. Lindmar Löffelholz and Muscholl 1968 Löffelholz and Muscholl 1970, Malik and Ling 1969 Vanhousette and Shepherd 1973). Furthermore, peripheral adrenergic and cholinergic axons have been found to run concomitantly and tightly intertwined in several tissues (Eränkö and Räsänen 1965 Ehinger and Falck 1965 Jacobowitz and Koelle 1965 Tränzer and Thoenen 1967 Ehinger Falck and Sporrang 1970) Finally the possibility should be kept in mind that the cholinesterase-positive nerves are the fibres secreting the inhibitory transmitter perhaps together with acetylcholine although the transmitter could be secreted from a third kind of fibre as well.

The relaxing effect seen with nicotine and DMPP and sometimes with high concentrations of acetylcholine is probably due to stimulation of nicotinic receptors in the inhibitory nerves. This assumption is based on the finding that the effect is blocked by ganglionic blocking agents as well as by lidocaine. The point of attack of nicotine is postganglionic rather than ganglionic since no autonomic ganglia have been observed in the retractor penis of several species (Fletcher 1897, Fischer 1917, Klinge, Pohio and Solatunturi 1970). This was

be in accordance with earlier findings showing that nicotinic drugs activate pre- and postganglionic C fibres (e.g. Armitt and Ritchie 1961, Schaeppi, Dennison and Dodd 1966). In analogy the small and rapid contraction sometimes preceding the inhibition may be due to nicotinic activation of excitatory adrenergic nerve fibres, and this could also apply to the contractions of the dog retractor penis brought about by nicotine in the studies of Edmunds (1920) and Luduena and Grigas (1966). The relaxations of the dog retractor penis produced by large concentrations of acetylcholine in the studies of Orlov (1963 b) and Gushchin (1965) were presumably due to nicotinic activation of the inhibitory nerves.

In this study any attempt to evaluate the validity of the original Burn and Rand hypothesis has deliberately been omitted. Accordingly we are unable to draw any conclusions although some findings might provide indirect evidence in favour of the theory as for instance the effects of botulinum toxin and physostigmine. Alternative explanations as direct or indirect postjunctional effects of physostigmine and a direct effect of high concentrations of botulinum toxin on adrenergic neurotransmission (Holman and Spitzer 1973) are, however, as possible. Acetylcholinesterase activity has been demonstrated in sympathetic adrenergic axons (Eränkő et al. 1970). So far the possible existence of acetylcholinesterase-positive acetylcholine-containing adrenergic fibres in the bull retractor penis can not be excluded. But if this is true then the question is, why are not all adrenergic fibres cholinesterase-positive?

Posterior pituitary hormones

Earlier findings have suggested that posterior pituitary hormones relax the bull and dog retractor penis (Klinge 1970 c, Luduena and Grigas 1972) and dilate the pudendal artery and its branches (Holmquist and Olén 1968). In the cited studies the hormones were stored in organic solvents. The present results indicate that the solvents alone were responsible for the relaxations observed. The mere hormones had no effect on the retractor penis and contracted penile artery. It is unlikely that posterior pituitary hormones locally contribute to the establishment of penile erection. The considerably stronger vasoconstrictor effect of the bovine arginine-vasopressin in comparison with the porcine lysine-vasopressin further supports the above view.

Some final remarks

The exact vascular events in penile erection of the bull are poorly understood. The possible importance of vasocompression due to activity of the ischiocavernosus muscle has been stressed by Watson (1984). The enormous peak pressure, 1727 mm Hg, in the corpus cavernosum penis during erection reported by Lewis et al. (1968) indicates that erection in the bull may not just be a simple matter of difference between in and outflow resistance of the cavernous bodies. Provided this value gives a correct picture of the pressure within the thick and fibrous tunica albuginea an active vasocompression must take place. The theory of active vasocompression as well as the correctness of the pressure values reported for the corpus cavernosum penis of the bull are indirectly supported by the huge pressures recently recorded during erection in the corpus cavernosum penis of the goat and the stallion (Beckett et al.

1972a, b and 1973). Such pressure could be possible only in species in which the penis is covered by a specially thick tunica albuginea. The recent electrophysiological studies of Hart (1972) indicate that also in the dog adjacent striated muscles play a role in the establishment of complete erection. An active compression effectuated by the adjacent striated muscles does, however, exclude that dilation of the inflow resistance vessels of the cavernous bodies is a prerequisite for full penile erection. In the termination of erection and establishment of the sigmoid flexure of the bull penis the structural architecture of the fibrous elements are probably of importance (Ashdown *et al.* 1962, Ashdown and Smith 1969).

The bull retractor penis is a smooth muscle which obviously can be kept in tone by a few excitatory nerve impulses but which also is promptly relaxed by a limited number of inhibitory impulses. The mere cessation of the inhibitory discharge may lead to replacement of relaxation by a strong rebound contraction. Thus the muscle is well adapted for its task, i.e. to permit an almost instantaneous protrusion of the penis during copulation and to produce a rapid withdrawal of the penis after copulation (cf. Ashdown and Pearson 1973). In contrast to its slow endogenous activity the smooth muscle is very efficient as a neuroeffector unit. Previously it has been pointed out that the effectors of seminal emission, i.e. the smooth muscle of the vas deferens, seminal vesicle and prostate with their abundant adrenergic supply are well adapted for brief and forceful contractions (Sjöstrand 1965) thus facilitating rapid ejaculation (cf. Seldel and Foote 1969). In the case of the smooth muscle effectors of erection the autonomic neuroeffector units are constructed for prompt relaxations as well. The physiological significance of this is that the time of copulation can be short. With some exceptions, as e.g. domestic hog and also dogs, most mammals have very short copulation times. For animals of prey as e.g. the bovidae, a short copulation time may be of advantage with respect to the exposure to predators. Among other things reproduction and reproductive behaviour involves an increased risk for capture of the animal.

Finally the validity of the present results for species other than ox should be considered. From the reviewed literature (Chapter I) it seems likely that the smooth muscle effectors of erection in other species also have an excitatory adrenergic innervation. Although under disputable experimental conditions, penile erection has been reported to be atropine-fast in boar, dog, cat, rabbit and also in guinea-pig. It is thus possible that at least in these species erection may in part be effectuated by a non-cholinergic neurotransmitter. Presumptive cholinergic fibres may however participate directly in the smooth muscle relaxation of erection in some species. Thus cholinesterase-positive fibres are present in the smooth muscle of the cavernous bodies of the rabbit and this muscle is relaxed by acetylcholine (cf. Chapter I). It can of course also not be excluded that in certain species presumptive cholinergic fibres could be motor fibres.

SUMMARY

The effects of field stimulation and various endogenous compounds and drugs affecting the functions of autonomic nerves or receptors were investigated on isolated strips of the retractor penis muscle and the penile artery of the bull.

The following main results were obtained

1) The retractor penis exhibited rhythmic spontaneous activity. Spontaneous activity was also seen in some penile artery preparations. Sustained tonic contractions were frequent in both smooth muscles.

2) Field stimulation of the smooth muscles induced a monophasic, biphasic or triphasic response. If the strips were in low tone a motor response dominated or was the only response observed. If the strips were in high tone a relaxation dominated or was the only response observed. In preparations being in medium stage of tone, the response to field stimulation was contraction followed by relaxation. In the retractor penis the relaxation was often followed by a secondary contraction. This was rare in penile artery strips.

3) The frequency response relationship of the motor response to field stimulation indicated a maximum response at high frequencies. However in the retractor penis strong contractions could be elicited by single shocks. Trains of stimuli delivered at low frequencies could maintain the muscle in sustained contraction.

4) In the penile artery the frequency response relationship of relaxation induced by field stimulation was similar to that of excitation. In the retractor penis the inhibitory response to field stimulation was characterized by a rather sharply delineated maximum occurring at a limited number of pulses.

5) The secondary contraction was mainly dependent on the grade of the inhibitory response to field stimulation. Its peak occurred 2—3 min after cessation of stimulation.

6) The excitatory and the inhibitory responses to field stimulation as well as the secondary contraction were all abolished by tetrodotoxin or local anaesthetic drugs.

7) The excitatory response to field stimulation was inhibited or abolished by α -adrenoceptor and adrenergic neuron blocking agents. It was enhanced by inhibitors of neuronal noradrenaline uptake.

8) Noradrenaline and adrenaline contracted the retractor penis and the penile artery. This effect was abolished by α -adrenoceptor blocking agents. After α -receptor blockade adrenaline and noradrenaline produced relaxation. So did also isoprenaline. These relaxations were prevented by β -adrenoceptor blocking agents.

9) The inhibitory response to field stimulation was not prevented by anti-muscarinic, ganglionic blocking or neuromuscular blocking drugs. Nor was it counteracted by botulinum toxin or hemicholinium. It also appeared to be unaffected by physostigmine. It was uncovered by adrenergic neuron blocking agents.

10) Three different effects of acetylcholine were noted. a) contraction of the smooth muscle, b) suppression of the excitatory response to field stimulation, and c) a brisk short lasting relaxation. This relaxation was sometimes preceded by a rapid contraction and resembled the effect of transmural nerve stimulation. The first two effects of acetylcholine were emulated by pilocarpine and prevented by antimuscarinic drugs. The third effect was prevented by hexamethonium and emulated by nicotine. Nicotine-induced relaxations were prevented by ganglionic blocking agents as well as by local anaesthetics. All three acetylcholine effects, particularly the last one, required high concentrations.

11) The retractor penis and the penile artery were contracted by histamine and 5-hydroxytryptamine. The inhibitory response to field stimulation was not blocked by antihistamines or serotonin antagonists.

12) The retractor penis was contracted by ATP. The penile artery was relaxed by ATP. This relaxation was abolished or reversed after desensitization to ATP. This treatment had no effect on the inhibitory response to field stimulation. A number of other nucleotides and nucleosides were without effect or had weak effects similar to those of ATP.

13) No overt effects on the retractor penis and the penile artery were obtained with GABA, glycine, glutamic acid, aspartic acid or several other amino acids.

14) Prostaglandins E_1 and E_3 relaxed the retractor penis, whereas prostaglandin F_{2a} contracted it. All these prostaglandins were powerful stimulants of the arterial smooth muscle. Prolonged exposure to inhibitors of prostaglandin synthesis did not suppress the inhibitory responses to field stimulation.

15) Minute concentrations of bradykinin contracted the retractor penis. This peptide had almost no effect on the penile artery. Substance P contracted both muscles. Posterior pituitary hormones had no overt effect on the retractor penis but contracted the penile artery.

CONCLUSIONS

1) The smooth muscle of the retractor penis and probably also that of the penile artery of the bull possess certain characteristics of single unit smooth muscle.

2) The motor nerves of these smooth muscles are adrenergic. A very low tonic discharge in the adrenergic nerves is sufficient to maintain the muscles in sustained contractile activity.

3) In these smooth muscles there also are inhibitory nerves. The neurotransmitter which is released from the inhibitory nerves and which relaxes the muscle cells by acting directly upon them is most probably a substance other than acetylcholine. It is further unlikely that it is histamine, 5-hydroxy tryptamine, a prostaglandin or any of the nucleosides, nucleotides or amino acids studied. In the retractor penis a discharge in the inhibitory nerves probably leads rapidly to a high degree of occupancy of receptors for the inhibitory neurotransmitter. This may be of importance for erection.

4) The secondary contraction observed after cessation of inhibitory nerve stimulation is probably a true rebound effect and not due to stimulation of a third kind of nerve fibres. The rebound contraction may be of importance for the termination of erection.

5) If cholinergic nerves are present in these smooth muscles, the most probable effect of the presumptive cholinergic fibres is inhibition of adrenergic neurotransmission. It is unlikely that they directly produce the smooth muscle relaxation of erection. Relaxation of the smooth muscles caused by acetylcholine is probably due to nicotinic stimulation of the inhibitory nerves. The mere interruption of tonic adrenergic discharge is not sufficient to produce full penile erection.

6) It is unlikely that posterior pituitary hormones contribute locally to the establishment of penile erection in the bull. This also applies to angiotensin, bradykinin, and Substance P.

7) The differences observed in the effects of certain compounds on the retractor penis and the penile artery are due to differences in the muscle cells rather than to differences in their innervation.

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